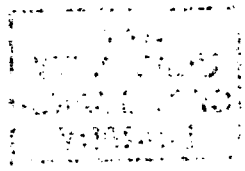

The Reproductive Biology of *Nerine* (Amaryllidaceae)

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B.Sc. (Hons). Dip.Ed.

submitted in fulfilment of the requirements
of the Degree of Doctor of Philosophy

University of Tasmania

October, 1999.

I declare that the material contained within this Thesis is original and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the Thesis. To the best of my knowledge and belief this Thesis contains no material previously published or written by another person except where due acknowledgement is made in the text of the Thesis.

Natalie Brown
14 October 1999

Natalie Brown
14 October 1999

Abstract

The genus *Nerine* (Amaryllidaceae) native to Southern Africa, comprises bulbous perennials with colourful, long-lasting blooms prized for cut-flower and bulb production. Hybridisation programmes have been undertaken since the late 19th century, breeding towards specific horticultural traits becoming more intensive in recent years. This work represents the first major study of the reproductive biology of *Nerine*, and provides a scientific basis for ongoing breeding work.

The inflorescence of *Nerine* is preformed within the bulb scales up to 32 months prior to anthesis, with floral development occurring continuously during this period. Sequential floret initiation occurs during the first growing season, followed by differentiation of the floral organs in year two. Gametogenesis occurs in the final months prior to anthesis, being asynchronous within an inflorescence. The lengthy period of floral development renders the inflorescence susceptible to disturbance over three growing seasons and two critical periods have been identified. Environmental conditions also act on the inflorescence at the time of scape emergence.

The fertilisation process from pollen germination to entry of the pollen tube into the micropyle takes approximately three days. The onset, duration and period of maximal pistil receptivity has been directly measured via seed set. However, this period does not correlate with indirect measures of receptivity. The length of the style at anthesis, relative to anther position, can be in one of three positions in *Nerine* (below, level or exsert). In all cases, elongation of the style occurs post-anthesis, with maximal elongation occurring in the short-styled cultivars. These short-style morphs have a greater rate of seed set from both open and self-pollinations.

The seeds of *Nerine* are fleshy and have no dormancy period. Following successful fertilisation, a nuclear endosperm develops and the integumentary tissue increases. At seed shed, a proembryo is present and the majority of embryo development occurs during after-ripening. Embryo development is influenced by temperature, with development and hence germination able to be suspended in some species by storage at 4°C. There was evidence of parthenogenetic seed production.

A hybridisation programme, operating at intra- and inter-specific and inter-generic levels, was initiated, with the fertility of seven species and 76 cultivars assessed. This yielded seeds from 96 intra-specific, eleven inter-specific and eight inter-generic crosses. In addition, self-compatibility has been established in five species and 23 cultivars. Pre- and post-zygotic barriers have been identified in failed crosses at all hybridisation levels, with a number of interventionist techniques being tested to overcome these barriers. *In vitro* culture of fertilised ovules was found to increase germination rates of hybrid seeds and protocols for *in vitro* pollination and embryo rescue have been developed. In addition, a successful technique for gynogenesis has been developed for use in *N. flexuosa* 'alba'.

Abstract (continued)

The hybridisation history of *Nerine* has resulted in the nomenclature of cultivars being in a chaotic state. As a result of this project, 1291 *Nerine* hybrids have been catalogued, with a collection of 95 *Nerine* cultivars described to allow assessment for horticultural purposes. Morphological characters have been assessed for use in cultivar identification schemes and for indicators of hybrid parentage. In addition a preliminary RAPD analysis has indicated this will be a useful tool for establishing relationships between cultivars and individual cultivar identification.

Acknowledgements

My supervisors, Dr. Ron Crowden, Dr. Janet Gorst and Dr Anthony Koutoulis for their expertise, guidance and encouragement throughout this research project.

My colleagues Diane Sward, for endless hours of meticulous scribing and Craig Gilmour for expert technical assistance with RAPD analysis, as well as for their valued friendship.

Members of the School of Plant Science, academic, technical and general staff and fellow students who have assisted me with my project and provided encouragement and support. In particular Dr. Yvonne Menadue for the use of her computer for the production of this thesis, Ilga Zodina-Hayde, Leanne Sherriff and Ivan Regan for getting me started in Tissue Culture and Microscopy.

Members of the University of Tasmania who have provided advice and technical assistance and members of other Universities and Research Institutions who have taken an interest in my project and given me valuable advice.

The Crowden family for allowing me unlimited access to the Nerines at Channel Bulbs, and for the many cups of tea.

My extended family for their unending support. In particular, my husband Frank for his patience, Simone for technical advice and occasional lab assistance, Thomas for enthusiastic assistance wherever possible and Nicholas for keeping us all smiling. I am also indebted to my parents, Dawn and (the late) Bill for their love, encouragement and excellent childcare.

I gratefully acknowledge the support of an APA scholarship, without which balancing family and study would have been impossible.

For my father

William James Brown

(2/1/1928 - 19/4/1999)

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Figures and tables with A as a prefix (*e.g.* Table A.7.2) can be found in the appendices at the conclusion of the relevant chapter.

Abbreviations used in this thesis

A	Anthesis
ABA	Abscisisc acid
ANOVA	Analysis of Variance (statistical technique)
DAP	Days after pollination
DH_2O	Distilled water
DOP	Direct Ovule Pollination
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide tri-phosphates
ESEM	Environmental scanning electron microscope
F_1	First filial generation
GA_3	Giberellic acid 3
IAA	Indole-3-acetic acid
IFC	Isolated floret culture
inositol	myo-inositol
NAA	α-napthaleneacetic acid
NaOCl	Sodium hypochlorite
OP	Open pollinated
PCR	Polymerase chain reaction
PGM	Pollen grain meiosis
PMC	Pollen mother cells
RAPD	Random Amplified Polymorphic DNA
SI	Self incompatible
SC	Self compatible
UI	Unilateral incompatibility
UV	Ultra-violet light

Tissue Culture Media (see Tables A.7.1, A.7.2)

ACM	Anther culture medium
ERM	Embryo rescue medium
MS	Murashige and Skoog's medium (Murashige and Skoog, 1962)
MM	Modified Monnier's Medium (see Table A.7.2)
Mt	Multiplication medium (see Table A.7.2)
UOC	Unpollinated ovule culture

Terminology

Time relative to anthesis is represented thus:
A = Anthesis; **A-x** = x days prior to anthesis; **A+x** = x days after anthesis.

Chapter One

General introduction

NERINE. Herb. In Bot. mag. T 2124 (1820); Bak. in FC. 6: 209 (1896); Soelch & Roessl. in FSWA. 150: 11 (1969)

Bulbous herbs, with or without short neck. Leaves filiform to strap-shaped, produced with or later than inflorescence. Flowers few - many in umbels. Peduncle slender or robust; spathe-valves 2, lanceolate. Perianth zygomorphic or regular, tube 0 or very short; segments narrowly oblanceolate, more or less crisped. Stamens declinate or erect arising from base of perianth segments; filaments filiform, sometimes appendiculate at base; anthers oblong, dorsifixed, versatile. Ovary subglobose, 3-lobed, with few ovules in each loculus; style filiform, straight or declinate, obscurely tricuspidate at stigmatose apex. Capsule subglobose, with wall bursting irregularly; seeds 1 - few in each loculus, globose, often viviparous.

(Dyer, 1976)

1.1 Current taxonomic status

The genus *Nerine* is a member of the Class Monocotyledonae, Order Asparagales, Family Amaryllidaceae, Tribe Amaryllideae (Dahlgren *et al.*, 1985; Bremer *et al.*, 1998). The composition of the family and the position of the genus within the family has altered with successive taxonomic reviews (Tables A.1.1, A.1.2). The most recent by Snijman and Linder (1996) places *Nerine* in the sub-tribe Amaryllidinae along with *Amaryllis*, *Brunsvigia*, *Carpolyza*, *Crossyne*, *Hessea* and *Strumaria*.

Fifty-three species of *Nerine* have been described, the first *N. sarniensis*, under the nomenifer *Amaryllis*, by Linnaeus in 1753 (Table A.1.3). *Nerine* was raised to

generic rank by Herbert in 1820 (Baker, 1888) who subsequently described nine species (Herbert, 1821 cited in Traub, 1967). Major reviews of the genus have been undertaken by a number of researchers (Table A.1.4), the most recent being those of Traub (1967), Norris (1974) and Obermeyer (1993). The review of Traub (1967) assigned 30 species to four sections on the basis of morphological characters while Norris (1974) ascribed 31 species to 12 groups. The current status of the genus comprises 23 species (Snijman, 1995) with many previously described species being assigned varietal status (Obermeyer, 1993) (Table A.1.4).

1.2 Geographical distribution

All species of *Nerine* are native to Southern Africa (Norris, 1974). The genus is widespread with species occupying a range of habitats (Fig. A.1.1; Table A.1.4). Individual species range in abundance from the widespread *N. laticoma* to the localised *N. marincowitzii* known only in the Koup Karoo (Snijman, 1995; Hardman, 1996) (Figs. A.1.2 a-t). Habitats are diverse ranging from the cool Drakenburg Mountains, subject to snow and frost (*N. bowdenii*); swamps (*N. platypetala*); and desert plains ascending to mountains (*N. flexuosa*) (Norris, 1974; van Benschop and Brenk, 1993; Snijman, 1995). Individual geographical distribution maps are included in Figures A1.2 a-t.

1.3 Growth phenology

Plants of the genus *Nerine* are mainly autumn flowering and exhibit one of three basic growth strategies (Norris, 1974): (i) summer dormancy (the term dormancy as used herein denotes the period when no above-ground leaves are present.), where leaves emerge after, or concurrent with, flowering and persist over winter; (ii) winter dormancy, where leaves emerge in spring and senesce after, or just prior to, flowering in autumn; or (iii) evergreen habit, with leaves present throughout the year. Of these, winter dormancy is the most common (Table 1.1).

Growth occurs in developmental cycles, consisting of a series of leaf primordia, followed by the initiation of an inflorescence bud (Rees, 1985; van Brenk and Benschop, 1993). In most species these growth cycles occur annually. An unusual feature of *Nerine* is the presence of more than one inflorescence bud in the bulb at any one time, and at least in *N. bowdenii* and *N. sarniensis*, these buds have typically

been initiated one year apart. After initiation, buds require over two years of growth and development before flowering will occur (Theron and Jacobs, 1994; Vishnevetsky *et al.*, 1997; Chapter 2).

Summer Dormant	Winter Dormant			Evergreen
N. humilis	*N. appendiculata*	*N. gracilis*	*N. pancratoides*	*N. angustifolia*
N. pudica	*N. bowdenii*	*N. hesseoides*	*N. platypetala*	*N. filifolia*
N. sarniensis	*N. frithii*	*N. huttoniae*	*N. rehmanii*	*N. flexuosa*
	N. filamentosa	*N. krigei*	*N. transvaalensis*	*N. masonorum*
	N. gaboronensis	*N. laticoma*	*N. undulata*	
	N. gibsonii	*N. marincowitzii*		

Table 1.1: Growth habit of *Nerine* species (after Norris, 1974; Saunders, 1997).

1.4 History of *Nerine* in cultivation

N. sarniensis was named for bulbs found naturalised on the island of Guernsey (Latin = Sarnia), and is commonly known as the 'Guernsey Lily'. These plants, established prior to 1700, are thought to be from the wreckage of a Dutch trading ship returning from the East via the Cape of Good Hope. Such traders, who used Table Bay as a supply port, almost certainly introduced the genus to Europe, with South African plants present in the Botanical Gardens at Leiden, Amsterdam in the early 17th century (Traub, 1967; Smithers, 1990). The first *Nerine* bulb was described as "Narcissus japonicus flore rutilo" by Cornut in 1635 (van Brenk and Benschop, 1993). Specimens were found in London by 1659 (Ammal, 1951) and Paris by 1666 (van Brenk and Benschop, 1993).

Following British colonisation, early Governors of the Cape were responsible for a number of botanical expeditions. In particular, Ryk Tulbagh (Governor 1751-71) corresponded with Linnaeus, sending him many specimens. Early botanical collections were made by Thunberg, a pupil of Linnaeus and Francis Masson, of the Royal Botanical Gardens, Kew (Leighton, 1939). Masson published descriptions of the many species collected in *Hortus Kewensis* (1789). In the late 18th and early 19th century, several systematic collectors journeyed into the interior of South Africa including Patterson, Burchell, Drege and Harvey (Leighton, 1939). The results of

these forays were extensive herbaria and a number of publications, most notably *Flora Capensis* in 1840 (Table A.1.6).

The horticultural potential of *Nerine* with attractive, long-lasting blooms has long been recognised with hybridisation programmes initiated in the mid-late 19th century. The first named hybrids were raised by Dean Herbert and by the time of publication of J.G. Baker's monograph, Amaryllidaceae (1888), 20 hybrids were listed. From these beginnings, the number of named hybrids has continued to grow, with significant collections of *Nerine* cultivars being in existence in the early 1900s.

A number of *Nerine* enthusiasts have continued hybridisation programs throughout the 20th century, further increasing the available number of cultivars. These include H.J. Elwes, Messers Barr and Sons, the de Rothschild Family, Mr Tony Norris, and Sir Peter Smithers (U.K); Mrs Emma Meninger and Mr Charles Hardman (USA); Mr. Hirao (Japan); and Mr Richmond Harrison and Mr Monty Hollows (New Zealand). Named hybrids presently number well over 1000 (Appendix A: Review of *Nerine* cultivars).

In Australia, varieties of *Nerine* were grown from the early 20th century, as Australian conditions, particularly those of the southern states (Tasmania and Victoria) were well suited for the growth of these plants (Cowlishaw, 1935). Hybidisation programs for improved varieties were also undertaken with the well known cultivar *Nerine* x 'Old Rose' originating from Australia.

In the latter decades of the 20th century, the colourful and long-lasting blooms of *Nerine* have become popular, not only with collectors and gardeners, but as cut-flowers. Consequently, commercial cultivation of *Nerine* now occurs throughout the world, in particular The Netherlands, Israel and South Africa, with fledgling industries in New Zealand and Southern Australia.

1.5 Horticultural perspective

Plants of the genus *Nerine* are used for commercial cut-flower and bulb production, a usage which is currently expanding world wide (Coertze and Louw, 1990; Smithers 1990). A possible future use is the production of alkaloids that are present in these plants (Traub, 1967).

The cultivars of *N. bowdenii* are currently grown in the greatest number due to their amenability to horticulture, being hardy and having a tolerance of cold temperatures allowing them to be grown outdoors in Europe and North America (Genders, 1973; Smithers, 1984). The bulbs can also be lifted and stored to allow extension of the flowering season. Although popular, the range of colours is limited in *N. bowdenii* and the inflorescence is not as well formed as in the cultivars of other species such as *N. sarniensis*. Cultivars of *N. sarniensis* range in colour from white through pink, red, orange and mauve with well formed inflorescences that are well-received as cut-flowers. However, *N. sarniensis* cultivars are much more difficult to grow than *N. bowdenii* cultivars. In particular, irregular flowering has prevented their widespread use as a cut-flower crop (Douglas, 1967; Smithers, 1984; van Brenk and Benschop, 1993). Short scape length as well as slow multiplication rates are other common problems in *N. sarniensis* and limits the use of these cultivars in commerce. Additionally, to be grown in Europe, bulbs of *N. sarniensis* require heating (Smithers, 1984), because they are susceptible to the harsh European winter frosts, hence needing to be grown under glass in winter. In theory, this could be solved by moving material into glasshouses for the winter season, however, in practice this is more difficult due to the sensitivity of the plant to root damage, and the inflorescence to disturbance (see Chapter 3).

Although there have been a number of *Nerine* breeding programmes, these have mainly been the work of enthusiasts attempting to produce aesthetically pleasing or novel hybrids for collectors. Unfortunately, very few parentage details are documented from previous work. Notable exceptions include the early breeding of Dean Herbert, the Exbury programme and the breeding programmes of Mr Tony Norris (records now held by Mr Monty Hollows) and Sir Peter Smithers (see Chapter 10). Furthermore, many valuable breeding varieties have not been preserved over the years. Nevertheless, hybridisation is being continued throughout the world using the significant variation present in the genus (Coertze and Louw, 1990; van Brenk and Benschop, 1993).

The quest for commercially valuable cultivars has become more serious in recent years and the emphasis has changed to breed varieties with specific horticultural traits. An example is the substantial export market being developed in New Zealand by Nerine Nurseries Ltd., who are developing cultivars tailored to the Japanese wedding market. Continuing research and development is concentrating on producing longer stemmed varieties for the US market (Anon., 1995 J. N.Z. flower industry). Commercial hybridisation programmes have also operated in Israel and Netherlands, but the number of commercial lines produced so far has been limited.

Many of the past hybridisation programmes have used *N. sarniensis* cultivars, due to the superior form of the inflorescence and the greater range of available colours. Some usage of cultivars of other species, such as *N. bowdenii,* has been documented. *N. bowdenii* has a cultivation advantage, particularly in cooler climates, as it can be grown outdoors. Additionally, *N. bowdenii* is a more reliable producer of flowers than the irregularly flowering *N. sarniensis*. Inter-specific crosses between these two species have been successful (see Chapter 5), but breeding thus far has rarely gone beyond the first generation due to problems with hybrid sterility (see Chapters 6, 7). Use of the less common *Nerine* species in inter-specific crosses has been limited to date (see Chapter 6). Thus, the potential of these species for the introduction of desirable characteristics remains to be assessed.

Inter-generic hybridisation together with polyploidisation is the focus of present breeding research in the Netherlands, with financial support from the Dutch *Nerine* growers (Toussaint, 1995). In New Zealand, The Horticulture and Food Research Institute of New Zealand Ltd. (HortResearch) are researching techniques such as somacloning and recombinant DNA as possible methods of producing improved cultivars (Beauchamp, 1990). Additionally, research into the growth physiology of *Nerine* is being undertaken in New Zealand (HortResearch), the Netherlands (Waegeninen) and South Africa.

Two further difficulties of *Nerine* cultivation are the low multiplication rates and long periods from seed to first flower. These problems have inspired horticulturists to search for alternative methods for bulking up new varieties. Tissue culture, as a means of rapid cloning, has been developed for a number of cultivars (Pierik and Steegmans, 1986; Custers and Bergervoet, 1992; Jacobs *et al.*, 1992; Lilien-Kipnis *et al.*, 1992; Ziv *et al.*, 1994). Tissue culture methods are also being developed at the

University of Tasmania, Australia, for the production of high quality virus-free bulbs. In Tasmania, field studies are underway to determine optimum growing conditions, through mulching and shading as well as developing methods of extending the flowering season.

1.6 Justification for this project

Tasmania offers excellent prospects for the development of a *Nerine* cut-flower industry. The climate and soil are suitable for temperate zone bulb crops and the winters in coastal Tasmania, while cold, are not associated with prolonged and harsh frosts. It is possible in these conditions for *N. sarniensis* to be grown outdoors, year round, and to be left with minimal off-season maintenance for 4-5 years before bulbs need to be lifted and divided. In addition, the island geography presents a disease quarantine advantage for export markets.

A small *Nerine* growing industry (based around *N. sarniensis, N. bowdenii* and *N. flexuosa* 'alba') has built up in Tasmania, Victoria and South Australia, yet it is still in its infancy. Improvement of *Nerine* cultivars will be of great benefit to commercial growers around Australia who will ultimately be able to supply high quality products to northern hemisphere markets in the 'off season'.

This project has presented a unique opportunity to generate data on the reproductive biology and fertility of *Nerine* species to put *Nerine* breeding on a scientific basis. The information generated will be used in the establishment of a breeding programme for *Nerine* improvement in conjunction with the Tasmanian Company, Channel Bulbs. Channel Bulbs, who have supplied all plant material used in this study, hold a significant collection of *Nerine* species and cultivars together with a growing collection of related Amaryllid genera (Plate 1.1). The ultimate aim of the *Nerine* improvement project will be to produce high quality bulbs targeted to fill niches in the international market.

1.7 Plant material used in this study

All plants used in this study were grown under cultivation at Channel Bulbs, Kettering, Tasmania, Southern Australia (Fig. A.1.3). The collection consisted of nine species and 95 cultivars (Table A.1.7). Representatives of related genera;

Amaryllis, *Brunsvigia*, *Cyrtanthus* and *Lycoris* were also held in the collection (Table A.1.5). At least one representative of each cultivar in the collection was grown under plastic tunnels (Plate 1.1). Additionally, field crops of *N. bowdenii* x 'Clone 63', *N. flexuosa* 'alba', *N. sarniensis* x 'Fothergillii major' and *N. sarniensis* x 'Rosea' were grown.

1.8 Limitations of this study

The most serious limitation of the present study was irregular flowering. Of the 104 varieties in the collection, only nine flowered in all seasons of the study (Table A.1.7). To compound this problem, only five of these were fertile, with a sixth exhibiting limited fertility (*N. sarniensis* x 'Fothergillii major', a known triploid). This caused difficulties as crosses could not be repeated and interventionist techniques could not be employed in known incompatible or incongruent crosses. Nevertheless, the continuous observation of growth and flowering during the study, in conjunction with monitoring of environmental conditions, has led to some insight into the causes of irregular flowering which will be extremely useful to *Nerine* growers (see Chapters 3, 8).

A second major difficulty arose from the chaotic state of cultivar nomenclature (see Chapter 9). Whether names of cultivars held in the collection are in agreement with those adopted elsewhere is unknown, as no reference source is available. It is hoped the work published herein will begin to overcome this problem, and the comprehensive documentation of characters and a photographic record may provide a base on which other breeders can build.

Finally, due to the long generation time of *Nerine* (3-5 years) I have been unable to assess any hybrids that have been produced as a result of this work. Nevertheless, breeding records will allow this assessment as the programme continues.

Plate 1.1: A section of the Channel Bulbs *Nerine* collection growing under a plastic tunnel at Kettering, Tasmania, Australia.

Author	Year	Summary of classification	Ref
Linnaeus	1758	*Nerine* included as *Amaryllis*. 1 species only *A. sarnienis*.	10
Michel Adanson	1763	Amaryllids included under family name Narcissi.	8
A.L. de Jussieu	1789	Amaryllids included under family name Narcissi.	8
Jaume St Hilaire	1805	Included under family name Amarylleae; 3 divisions.	8
R. Brown	1810	Amaryllidaceae: Division II of St. Hilaire (inferior ovary)	8
Lindley	1853	Class Endogens. Order Narcissales. Family Amaryllidaceae (included Agavaceae and Alstromeriaceae).	5
Bentham and Hooker	1883	Series Epigynae. Family Amaryllidae.	5
Pax	1887	4 subfamilies. Amryllidoideae: 53 genera. Family included Agavoideae, Hypoxidoideae.	5
J. G Baker	1888	3 subfamilies; Amarylleae, Alstromeriaceae, Agavaceae.	1
Van Tiegham	1891	Order Iridinees, included Amaryllidees. (Taccaceae, Velloziaceae and Burmanniaceae included).	5
Engler	1892	Order Liliflorae. Family Amaryllidacae. (Taccaceae, Velloziaceae and Burmanniaceae removed).	5
Wettstein	1901	Order Liliflorae. Family Amaryllidacae. (Included Agavaceae, *pro parte* Hypoxidaceae).	5
Lotsy	1911	Order Liliflorae. Family Amaryllidacae. (Agavaceae and Hypoxidaceae removed).	5
F. Pax and K. Hoffman	1930	4 subfamilies, 86 genera.	5
J. Hutchinson	1935	Order Amaryllidales. Family Amaryllidaceae. (Included Allieae, Agapantheae and Gilliesieae). 13 tribes.	7,8
H.P. Traub	1938	14 Tribes: Alstromeraceae, Agavaceae not included	8
Kimura	1956	Amaryllidales. Family Amaryllidaceae (Included Alstromeriaceae and Hypoxidaceae).	5
H.P. Traub	1957	3 sub-families, 2 infra-families, 14 tribes, 68 genera, 930 species.	8
Takhtajan	1959	Order Liliales. Family Amaryllidaceae. (Alstromeriaceae and Hypoxidaceae removed).	5
H.P. Traub	1962	3 sub-families, 15 tribes (Included Allioideae, Allieae, Agapantheae and Gilliesieae).	9
Dahlgren	1975	Lilianae. Family Amaryllidaceae separate from Agavaceae, Alliaceae and Hemerocallidaceae.	4
Dahlgren, Clifford and Yeo	1985	Order Asparagales, Amaryllidaceae separate from Alliaceae and Gilliesiaceae. 13 tribes.	6
Gibbs, Russel, Reed, Van Roy and Smook	1985	17 genera, 212 species	3
Bremer, Chase and Stevens	1998	Order Asparagales. Family Amaryllidaceae. (Agavaceae Alstromeriaceae Hemerocallidaceae, Hypoxidaceae retain familial status)	2

Table A1.1: Taxonomic position of the family Amaryllidaceae.

References:

1 = Baker (1888).
2 = Bremer, Chase and Stevens (1998).
3 = Coertze and Louw (1990)
4 = Dahlgren (1975).
5 = Dahlgren and Clifford (1982).
6 = Dahlgren, Clifford and Yeo (1985)
7 = Hutchinson (1935).
8 = Traub (1957).
9 = Traub (1962).
10 = Traub (1967).

Author and year	Family structure*	Position of *Nerine*	Genera allied to *Nerine*
St-Hilaire (1805)	3 Divisions 21 Genera	Division II (inferior ovary)	*Haemanthus, Pancratium, Narcissus, Eustephia, Leucojum, Galanthus*
Baker (1888)	3 Sub-families 61 Genera	Sub-family Amarylleae; In 1 of 3 tribes	*Galanthus, Leucojum, Lapiedra, Hessea, Carpolyza, Gethyllis, Apodoliron, Cooperia, Anoiganthus, Childanthus, Sternbergia, Haylockia, Zephyranthes, Sprekelia, Ungernia, Lycoris, Hippeastrum, Vallota, Cyrtanthus, Griffinia, Clivia, Haemanthus, Buphane (Boophone), Crinum,Amaryllis, Ammocharis, Brunsvigia, Strumaria*
Hutchinson (1934)	13 Tribes	Tribe Amaryllideae	*Amayllis, Brunsvigia, Ungernia*
Hutchinson (1935)	13 Tribes	Tribe Amaryllideae	*Ammocharis, Amaryllis, Anoiganthus, Brunsvigia, Crinum, Cyrtanthus, Vallota*
Traub (1936, 1938)	14 Tribes	Tribe Callicoreae	*Ammocharis, Brunsvigia, Callicore (Amaryllis), Crinum, Stenoliron*
Traub (1957)	3 Sub-families 98 Genera 1523 Species	Subfamily : Amarylloideae Infra-family: Crineaenae Tribe: Crininae	*Ammocharis, Boophone, Brunsvigia, Crinum, Cybistetes*
Traub (1962, 1963)	4 Sub-families 20 Tribes 105 Genera 1644 Species	Subfamily IV: Amarylloideae Infra-family: Amarylloidinae Tribe: Crineae	*Ammocharis, Boophone, Brunsvigia, Crinum, Cybistetes*
Traub (1965)			*Ammocharis, Boophone, Brunsvigia, Carpolyza, Crinum, Cybistetes, Hessea, Strumaria*
Traub (1967)		Tribe Crineae	*Ammocharis, Boophone, Brunsvigia, Crinum, Cybistetes, Hessea, Strumaria*
D. and U. Müller Doblies (1985)		Tribe Crininae	*Ammocharis, Boophone, Brunsvigia, Crinum, Cybistetes*
Snijman and Linder (1996)		Tribe Amaryllidinae	*Amaryllis, Brunsvigia, Carpolyza, Crossyne, Hessea, Strumaria*

Table A.1.2: Significant Revisions of the Family Amaryllidaceae. * For further details see Table A.1.1.
Reference Sources: Baker (1888); Hutchinson (1935); Snijman and Linder (1996); Traub (1938; 1957; 1962a; 1965; 1967).

Nomenclature of *Nerine* species 1753-1995

Name	Date first described	Publication details	First published as *Nerine*	Synonyms	Current status	Ref/s
N. sarniensis	1753	*Amaryllis sarniensis* L Sp. Pl. ed 1:293, 1753	*N. sarniensis* (L) Herb. Bot. Mag. Sub pl 2124, 1820	*A. dubia, A. jacquinii; Haemanthus sarniensis; N. cochinchinensis, N. insignis, N. jacquinii, N. profusa, N. sarniensis* profusa, *N. venusta* sarniensis	*N. sarniensis*	1,2,9
N. curvifolia	1797	*Amaryllis curvifolia* Jacq. Hort. Schoenbr. 1:33 pl 64, 1797	*N. curvifolia* (Jacq) Herb. App 19, 1821	*Imhofia glauca; N. sarniensis* var curvifolia	*N. sarniensis*	2,9
N.humilis	1797	*A. humilis* Jacq. Hort. Schoenbr. 1: 36 pl 69, 1797	*N. humilis* (Jacq) Herb. Bot. Mag. Sub pl 2124, 1820		*N. humilis*	1,2
N. flexuosa	1797	*A. flexuosa* Jacq. Hort. Schoenbr. 35 t 67	*N. flexuosa* (Jacq) Herb. App 19, 1821	*N. pulchella*	*N. humilus*	1,9
N. marginata	1797	*A. marginata* (Jacq) Hort. Schoenbr. 1:36 pl 69, 1797	*N. marginata* (Jacq) Herb. Amaryll. 238, 1837	*B. marginata; I. marginata; Elisena marginata*	*Brunsvigia marginata*	2,6,9
N. undulata	1797	*A. undulata* L Syst. Nat. ed 12; Mill. Hort. Kew, 352	*N. undulata* (L) Herb. App 19, 1821	*A. major, A. aucta; H. undulata; N. crispa, N. aucta*	*N. undulata*	1,2,9
N. plantii	1805	*N. plantii* O'Brien, Flora of Sylva 3: 122, 1805		*N. sarniensis* var plantii	*N. sarniensis*	2,9
N. venusta	1808	*A. venusta* Ker Gawl. Bot. Mag. pl 1090, 1808	*N. sarniensis* var venusta (Ker Gawl) Bak. Amaryll 100, 1888	*I. venusta; N. venusta* var sarninesis, *N. venusta* var minor, *N. sarniensis* var venusta	*N. sarniensis*	2,9
N. lucida	1810	*A. lucida* Burchell	*N. lucida* Herb. Amaryll, 283, 1837	*A. laticoma; B. lucida*	*N. laticoma*	2,9
N. corusca	1811	*A. corusca* Ker Gawl. Bot. Mag. sub pl 1430, 1811	*N. sarninesis* var corusca (Ker Gawl) Bak. Amaryll 100, 1888	*A. humilus; I. corusca; N. corusca, N. sarniensis* var Corusca	*N. sarniensis*	2,9

Name	Date first described	Publication details	First published as *Nerine*	Synonyms	Current status	Ref/s
N. fothergilli	1816	*A. fothergilli* Andr. Bot. Reposit. 163, 1816	*N. fothergilli* Roem. Monogr. Amaryll 4:107, 1847	*N. fothergilli*, *N. curvifolia* var fothergilli, *N. fothergilli* var major, *N. sarniensis* var curvifolia f. fothergillii	*N. sarniensis*	2,9
N. aucta	1819	*A. aucta* Tratt.	*N. aucta* M. Roem. Syn Monogr. Amaryll. 4:107, 1847	*N. undulata* var major	*N. undulata*	9
N. laticoma	1820	*A. laticoma* (Ker Gawl) Bot. Reg. 6: pl 497, 1820-1	*N. laticoma* (Ker Gawl) Th Dur & Shinz. Consp. Fl. Afr. 5; 256, 1893	*A. lucida* , *A. laticoma*; *I. laticoma*, *I. lucida*; *N. lucida*	*N. laticoma*	1,9
N. pulchella	1821		Herb. Bot Mag. 2124, 1821	*N. flexuosa* var pulchella	*N. humilis*	7,9
N. rosea	1821		Herb. Bot Mag. 2124, 1821	*N. venusta* var rosea, *N. sarniensis* var rosea	*N. sarniensis*	9
N. angustifolia	1868	*N. pulchella* var angustifolia Baker Saund. Ref. Bot. t329, 1868	*N. angustifolia* W. Watson Gard. Chron. 3:6, 1889	*N. pulchella* var angustifolia, *N. flexuosa* var angustifolia, *N. angulata*	*N. angustifolia*	1,9
N. angulata	1868		*N. angulata* Norris, 1974			7,9
N. pudica	1868		Hook f Bot Mag. pl 5901, 1871		*N. pudica*	1,2,9
N. filifolia	1881		Baker Bot. Mag. t 6547		*N. filifolia*	1,2
N. Elwesii	1886		Leichtlin Gard. Chron. (I)42, 1886	*N. pudica* var elwesii *N. sarniensis* var elwesii,	*N. pudica*	9
N. Moorei	1886		Leichtlin Gard. Chron. ns 26:681, 1886	*N. sarniensis* var moorei	*N. sarniensis*	1,9
N. rehmanii	1888	*Hessea rehmanii* Bak. Amaryll. 22, 1888	*N. rehmanii* L. Bolus ex Traub. Sth Afr. Gard. 20: 148, 1930		*N. rehmanii*	9
N. pancratoides	1891		Baker Gard. Chron, ii 576, 1891		*N. pancratoides*	1,3,9
N. appendiculata	1894		Baker Gard. Chron. ii 336, 1894		*N. appendiculata*	1,3,9
N. brachystemon	1896		Baker Fl. Cap. 6(2): 212, 1896		*N. appendiculata*	1,3,9

Name	Date first described	Publication details	First published as *Nerine*	Synonyms	Current status	Ref/s
N. duparquetiana	1896	*Imhofia duparquetiana.* Baill. Bull Linn Soc. Paris ii) 1132, 1894	Baker Fl. Cap. 6(2): 214, 1896		*N. laticoma*	1,3,9
N. huttoniae	1903		Schoenland Rec Alb. Mus. 1:49, 1903	*N. laticoma* subsp Huttoniae	*N. huttoniae*	1,9
N. Schlecteri	1903		Baker Bull. Herb. Boiss, 2nd Ser 3:665,1903		*N. bowdenii*	9
N. bowdenii	1904		W. Watson Gard. Chron. 36:365, 1904	*N. veitchii, N. excellens* tardiflora, *N. excellens* major tardiflora	*N. bowdenii*	1,9
N. veitchii	1911		Hort.	*N. bowdenii*	*N. bowdenii*	9
N. Ridleyi	1913		E.P. Phillips Ann. Sth Afr. Mus. 9:128, 1913		*N. bowdenii*	9
N. pusilla	1914		Dinter Neue. Pfl. Deutsch-Suedwest-Afr., p 48, 1914		*N. pusilla*	1,9
N. frithii	1921		L. Bolus. Ann. Bolus Herb. 3:79, 1921		*N. frithii*	1,9
N. transvaalensis	1928		L. Bolus Fl. Pl. Sth. Afr. 18 subpl 683, subpl 681, 1928	*N. frithii* 'L. Bolus'	*N. transvaalensis*	1,9
N. masonorum	1930		L. Bolus. S. Afr. Gard. 20:148, 1930		*N. masonorum*	1,9
N. krigei	1932		Barker. Sth Afr. Gar. 22:137, 1932		*N. krigei*	1,9
N. falcata	1933		Barker. Fl. Pl. Sth. Afr. 13: 511, 1933		*N. laticoma*	3,9
N. alata	1935		Barker Fl. Pl. Ath. Afr. 15 pl 563, 1935		*N. undulata*	3,9
N. alta	1935		Barker		*N. undulata*	1,3,4, 6
N. breachiae	1935		Barker. Fl. Pl. Sth. Afr. 15: 566, 1935		*N. humilis*	1,3,9
N. filamentosa	1935		Barker. Fl. Pl. Sth. Afr. 15: pl 561, 1935		*N. filifolia*	3,9

Name	Date first described	Publication details	First published as *Nerine*	Synonyms	Current status	Ref/s
N. gaberonensis	1935		C.E.B. Brenkamp& A.A. Obermeyer Ann Traansvaal. Mus 16:409, 1935		*N. gaberonensis*	9
N. parviflora	1935	*N. filifolia* var parviflora Barker. Fl. Pl. Sth. Afr. 15: 568, 1935	*N. parviflora* Traub Rev. of Genus Nerine, 1967		*N. filifolia*	7,9
N. peersii	1935		Barker. Fl. Pl. Sth. Afr. 15: 562, 1935		*N. humilis*	3,7,9
N. tulbaghensis	1935		Barker. Fl. Pl. Sth. Afr. 15: 565, 1935		*N. humilis*	3,7,9
N. gracilis	1937		Dyer. Fl. Pl. Sth. Afr. 17:679, 1937		*N. gracilis*	1,5,9
N. hesseoides	1938		L. Bolus. Fl. Pl. Sth. Afr. 18: 683, 1938		*N. hesseoides*	9
N. gibsonii	1968		K. H. Douglas. J. Sth. Afr. Bot. 34(1), 1968		*N. gibsonii*	1,7
N. platypetala	1971		McNeill. J. Sth Afr. Bot. 37(4) 207-208, 1971		*N. platypetala*	1,7
N. marincowitzii	1995	*N. marincowitzii* Snijman Novon 5:103-105,1995			*N. marincowitzii*	8

Table A.1.3: Nomenclature of *Nerine* species 1753-1995.

References:

1. Obermeyer In: Arnold and de Wet (1993)
2. Baker (1888)
3. Barker (1939)
4. Compton (1936)
5. Dyer (1976)
6. Goldblatt (1972)
7. Norris (1974)
8. Snijman (1995)
9. Traub (1967)

Section *	Species	Geographical Distribution	Map	Dormancy	Colour	Size (cm)	Ref
Nerine (1)	*N. sarniensis*	SW Cape	A1.2a	Summer	Red	20-50	1,3,4,6
Laticomae (2)	*N. krigei*	SE Transvaal, NE Orange Free State	A1.2d	Winter	Pink/Red	60	3,4,6
Laticomae (2)	*N. laticoma*	Namibia, North Cape, Western Transvaal, Orange Free State, Botswana	A1.2b	Winter	Pink or white	15-30	3,4,6
Laticomae (2)	*N. huttoniae*	East Cape	A1.2c	Winter	Pink-maroon	15-30	3,4,6
Laticomae (2)	*N. marincowitzii*	Cape Province (Karoo)	n/a	Winter	Pink	20	3,4,5,6
Bowdeniae (3)	*N. bowdenii*	East Cape, Natal	A1.2e	Winter	Rose-pink or white	30-70	1,3,4,6
Bowdeniae (3)	*N. humilis*	SW and S Cape	A1.2h	Summer	Pale-Deep pink	15-50	3,4,6
Bowdeniae (3)	*N. undulata*	East Cape	A1.2k	Winter	Pink	45	3,4,6
Bowdeniae (4)	*N. pudica*	SW Cape	A1.2i	Summer	Pink or white with red	25-35	3,4,6
Bowdeniae (5)	*N. pusilla*	Namibia	n/a	Unknown	White/rose	7-9	3,4,6
Bowdeniae (5)	*N. rehmanii*	Transvaal, Swaziland	A1.2j	Winter	White	15	3,4,6
Bowdeniae (5)	*N. filifolia*	East Cape, Transkei, Orange Free State, Swaziland, Mpumalanga	A1.2g	Evergreen	Mauve-pink or white	50	2,3,4,6
Bowdeniae (8)	*N. angustifolia*	SE Transvaal, Swaziland, East Cape, Natal	A1.2f	Evergreen	Pink	100	1,3,4,6
Appendiculatae (5)	*N. gaberonensis*	SE Botswana, NW Transvaal, North Cape	A1.2n	Winter	Pink	25	3,4,6
Appendiculatae (7)	*N. pancratoides*	Natal	A1.2s	Winter	White or pale pink	95	3,4,6
Appendiculatae (7)	*N. platypetala*	SE Transvaal	A1.2t	Winter	Pink/ rose red	40	3,4,6
Appendiculatae (9)	*N. appendiculata*	Natal, East Cape	A1.2l	Winter	Pale-deep rose pink	80	3,4,6
Appendiculatae (9)	*N. gibsonii*	East Cape, Transkei	A1.2o	Winter	Pink to white	30	3,4,6
Appendiculatae (9)	*N. masonorum*	Transkei	A1.2r	Evergreen	Pink	25	3,4,6
Appendiculatae (10)	*N. frithii*	Transvaal, Orange Free State, East Cape	A1.2m	Winter	Pink	20	3,4,6
Appendiculatae (10)	*N. gracilis*	Transvaal	A1.2s	Winter	Rose pink	20	3,4,6
Appendiculatae (10)	*N. hesseoides*	Orange Free State	A1.2t	Winter	Pink	17-25	3,4,6
Appendiculatae (10)	*N. transvaalensis*	Transvaal	n/a	Winter	Pink	20	3,4,6

Table A.1.4: Species of the genus *Nerine* (ex Obermeyer, 1993; Snijman, 1995), geographical location and description.
* Section: ex Traub [1967] (ex Norris [1974]).

References:

1 = Bryan (1989)
2 = James (1936)
3 = Norris (1974)
4 = Saunders (1997)
5 = Snijman (1995)
6 = Traub (1967)

Author	Year	Summary	Ref.
Jacquin	1797	5 species (as *Amaryllis*)	8
Ker-Gawler	1805	8 species (as *Amaryllis*	8
Herbert	1821	9 species *	8
Herbert	1837	9 species and 3 varieties *	8
Baker	1888	10 species and 18 hybrids	1
Baker	1893	15 species	8
Barr	1935	50 species and hybrids	7
Compton	1936	24 species	2
Traub	1967	30 species (4 sections) *	8
Goldblatt	1972	32 species	3
Norris	1974	31 species (12 groups) *	4
Obermeyer	1995	22 species	5
Snijman	1995	23 species	6

Table A.1.5: Summary of *Nerine* Classification. * Major reviews of classification of the genus.

References:

1 = Baker (1880)
2 = Compton (1936)
3 = Goldblatt (1972)
4 = Norris (1974)
5 = Saunders (1997)
6 = Snijman (1995)
7 = Traub (1957)
8 = Traub (1967)

Publication	Year	Author/Editor	Place of publication
Species Plantarum	1753	Linnaeus	London
Braunschweig	1755	L. Heister	London
Hortus Kewensis	1789	Unknown	London
Botanical Magazine	1787	William Curtis	London
Hort Schoenbr	1797	N.S. Jacquin	Unknown
The Botanist's Repository	1797-1814	H. Andrew	London
Les Lilacees	1802-1816	P.J. Redouté	Paris
Botanical Cabinet	1813-1833	Loddiges	London
Botanical Register	1815-1847	J.B. Ker [J.B. Gawler]	London
Thesaurus Botanicus	1819	V.L. Trattinick	London
Preliminary Treatise on Amaryllidaceae	1822	Dean Herbert	London
Genera of South African Plants		W. H. Harvey	London
Amaryllidaceae	1837	W. Herbert	James Ridgway and Sons, London
Flora Capensis	1840	W. H. Harvey and W. Sonder	London
Illustrated Dictionary of Gardening	1884-88	G. Nicholson	Unknown
Gardener's Chronicle	1885+	Unknown	London
Handbook of the Amaryllideae	1888	J. G. Baker	Geo. Bell & Sons, London
Flora and Sylva	1905	W. Robinson	
Monograph on Amaryllidaceae	1930	F. Pax and K. Hoffman In: Engler and Prantl	Pflanzenfamilien **15**, Liepzig
Herbertia/Plant Life	1934+	H.P. Traub (Founding ed.)	California
Reserches sur la famille des Amaryllidaces	1934	J. Gay	Am. Soc. Nat. **10**: 75-109
The Families of Flowering Plants II: Monocotyledons	1934	J. Hutchinson	Oxford University Press, New York
Coombs' South Africn Plants for American Gardens	1936	S.V. Coombs	F.A. Stokes & Co., New York
Revision of the Amaryllidaceae	1935	J. Hutchinson	New York
Flowering Plants of South Africa	1935	R.A. Dyer	Unknown
Grey's Hardy Bulbs Vol II	1938	C.H. Grey	London
Classification of the Amaryllidaceae	1957	H.P.T. Taub	Plant Life **15**: 76-83.
The families of Flowering Plants	1959	J. Hutchinson	New York
Classification of the Amaryllidaceae	1962	H.P.T. Taub	Plant Life **18**: 50-54
Review of the genus *Nerine* Herb.	1967	H.P.T. Taub	Plant Life **23**: 3-32.
Nerine Society Bulletin	1968+		U.S.A.
The genus *Nerine*	1974	C.A. Norris	Nerine Society Bulletin **6**
The genera of South African Plants 2	1976	R.A. Dyer	Dept. Agricultural Tech. Services, Pretoria
The families of the Monocotyledons	1985	R.H.T. Dahlgren, H.T. Clifford and P.F. Yeo	Springer-Verlag, Berlin
Nerine (In: Plants of Southern Africa: Names and Distribution)	1995	A. A. Obermeyer In: Arnold and de Wet (eds.)	Mem. Bot. Survey S. Africa: **62**
Phylogenetic relationships, seed characters, and dispersal system evolution in Amaryllideae (Amaryllidaceae).	1996	D.A. Snijman and H.P. Linder	Ann. Miss. Bot. Gardens **83**: 362-386

Table A.1.6: Major publications featuring *Nerine** 1753-1996, * *Nerine* is referred to as *Amaryllis*.

Reference Sources: Baker (1880); Hutchinson (1935); Pam (1942); Traub, (1957); Snijman and Linder (1995); Leighton (1939); Meninger (1960); Hayward (1937); Hayward (1955); Fernandes and Fernandes (1945); Norris (1974).

NAME	Flower Produced			
	1995	1996	1997	1998
SPECIES				
Nerine angulata	X	X	X	X
Nerine bowdenii	√	√	√	√
Nerine crispa	X	√	√	X
Nerine filamentosa	X	√	X	X
Nerine filifolia	X	X	X	X
Nerine humilis	X	X	X	X
Nerine masonorum	-	√	X	√
Nerine sarniensis	√	√	X	X
Nerine undulata	√	√	√	X
VARIETIES				
Nerine corusca	√	√	√	X
Nerine flexuosa 'alba'	√	√	√	√
HYBRIDS				
Afterglow	-	-	√	√
Ancilla	-	√	√	X
Angela Limerick	√	X	√	X
Aurora	-	√	√	√
Bagdad	-	√	X	√
Bennett Poe	-	-	X	X
Bettina	-	X	X	X
Brahms	√	√	X	X
Cameo Beauty	-	-	√	√
Canasta	-	√	X	X
Captain Dunne Cook	-	√	X	X
Caroline	-	√	X	X
Cherry Ripe	-	√	√	X
Chorister	-	√	√	X
Clone 63	√	√	√	√
Coral Queen	-	X	X	X
Cranfield	-	√	√	X
Cuckfield	-	-	√	√
Curiosity	-	√	√	X
Cynthia Chance	-	-	-	√
Donna	√	√	X	X
Dunkirk	-	-	X	X
Early Snow	√	√	√	X
Elvira	-	√	√	X
Enchantress	-	-	√	X
Erubescens	-	√	√	X
Eve	-	√	X	√
Evelyn Humphries	-	-	√	√
Flame	-	√	√	X
Flame Brilliant	√	X	√	√
Flame Spire	-	X	X	X
Fletcherii	-	-	-	√
Fothergillii	-	√	X	X
Fothergillii Major	√	√	√	√
Fred Danks	-	√	X	X
Galaxy	-	√	X	X
Gay Girl	-	-	X	X
Gladys Dettman	-	√	√	X
Gloaming	-	-	X	X
Gilbert Errey	-	√	X	X
Gold Dust	-	√	X	X
Guy Fawkes	-	√	X	X
Hera	-	-	X	√
Imp	√	√	X	X
Indian Orange	-	√	X	X
Jean O'Neill	-	√	X	X
Jenny Wren	-	√	X	X
Jill	√	√	√	√
Judith	-	-	-	√
Kentlworth	-	-	√	X
Killi	-	√	X	X
Knight Templar	-	-	X	X
Lady Lucy	√	√	√	X
Late Comer	√	X	√	X
Lucinda	-	-	√	X
Magenta Princess	√	X	X	X
Manselli	-	√	√	X
Mother of Pearl	-	√	X	X
Mrs Bromley	-	-	-	√
Mrs Cooper	-	√	X	X
Novelty	-	-	√	√
Old Rose	-	√	√	√
Optimist	-	-	√	X
Pink Bouquet	-	X	X	X
Pink Distinction	-	X	√	X
Pink Fairy	-	√	X	X
Pink Jewel (Aust)	√	√	√	√
Pink Opal	√	√	√	X
Pink Sensation	-	-	X	X
Pink Triumph	-	-	X	X
Radiant Queen	-	-	X	X
Redhead	-	-	√	X
Rosamund Elwes	-	-	-	√
Rosea (Aust)	√	√	√	√
Roseo-crispa	-	√	√	X
Rosita	-	-	√	X
Rose Princess	√	√	X	X
Rushmere Star	√	X	√	√
Salmon Supreme	-	√	X	X
Salmonea	√	√	X	√
Sarniensis profusa	-	-	X	X
Shelagh Mulholland	-	-	-	√
Snow Maiden	-	√	X	X
Splendens (A)	-	√	X	X
Splendens (B)	-	√	X	X
Sunset Falls	-	√	√	X
Trumpet's Blare	-	-	X	X
Western Sunset	-	√	X	X
White Dove	-	√	X	X
Winter Cheer	-	√	√	√
Virgo	-	√	X	X
Xanthia	-	√	X	X
OTHER AMARYLLIDS				
Amaryllis belladonna	-	√	√	√
A. belladonna 'Huthor'	-	√	√	√
Lycoris aurea	-	√	X	X
L. radiata	-	√	X	X
Cyrtanthus elatus	-	-	√	√

Table A.1.7: Flowering history of *Nerine* Cultivars and related amaryllids in the Channel Bulbs collection.

Key: √= Plant flowered;
X = No flower stalk produced;
- = Not present in collection

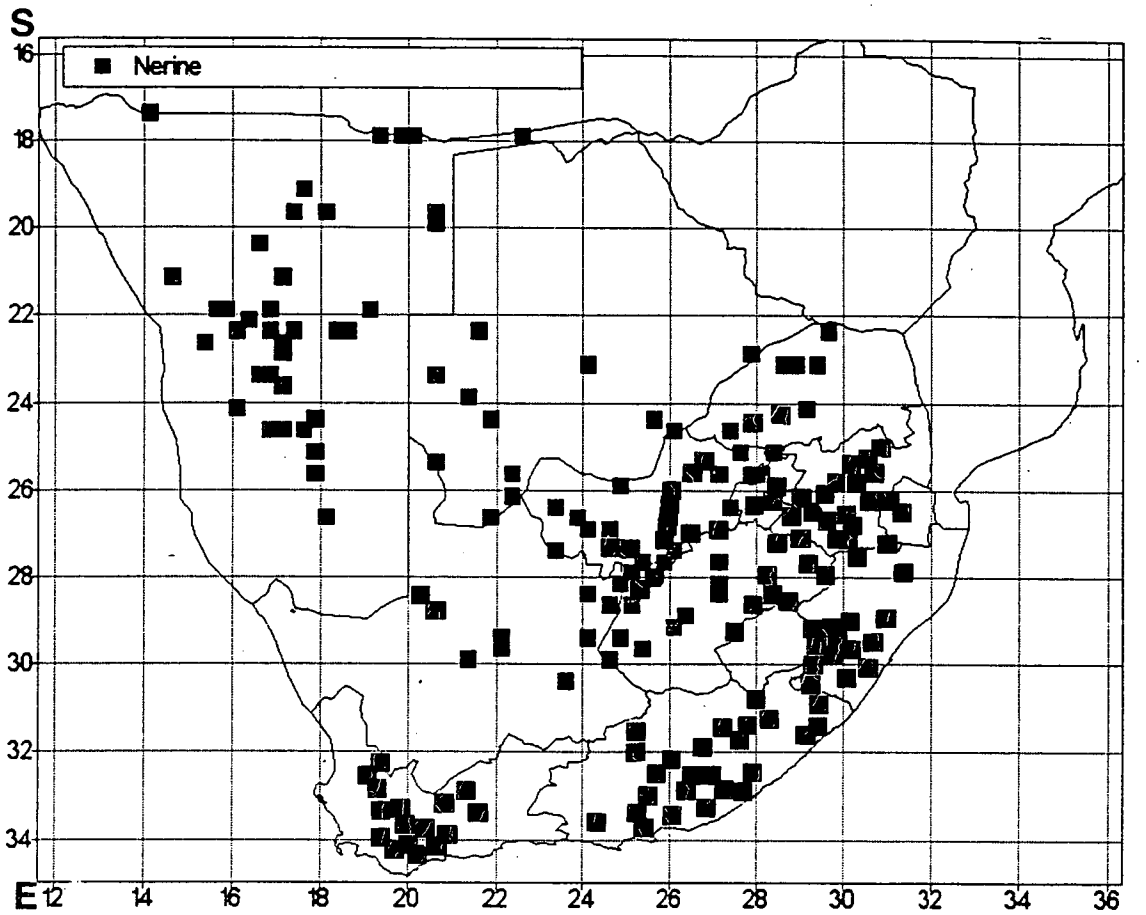

Figure A1.1: Geographical distribution[1] of the genus *Nerine* (Amaryllidaceae). This figure was collated from data supplied for individual *Nerine* species. For distribution of individual species see Figs A1.2 a-t.

[1] The National Botanical Institute is thanked for the use of the data from the National Herbarium, Pretoria (PRE) Computerised Information System (Precis).

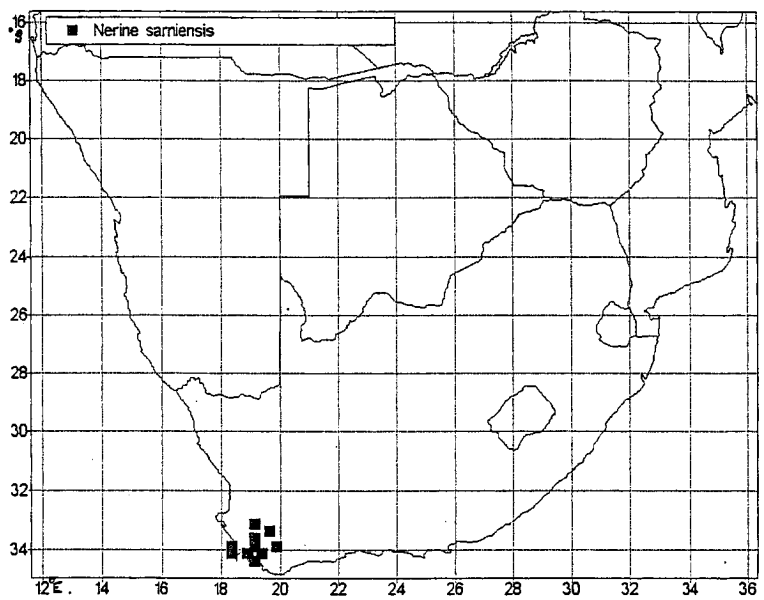

Figure A1.2 (a): Geographical distribution of *Nerine* species: Section Nerine (ex Traub, 1967) (a) *N. sarniensis*

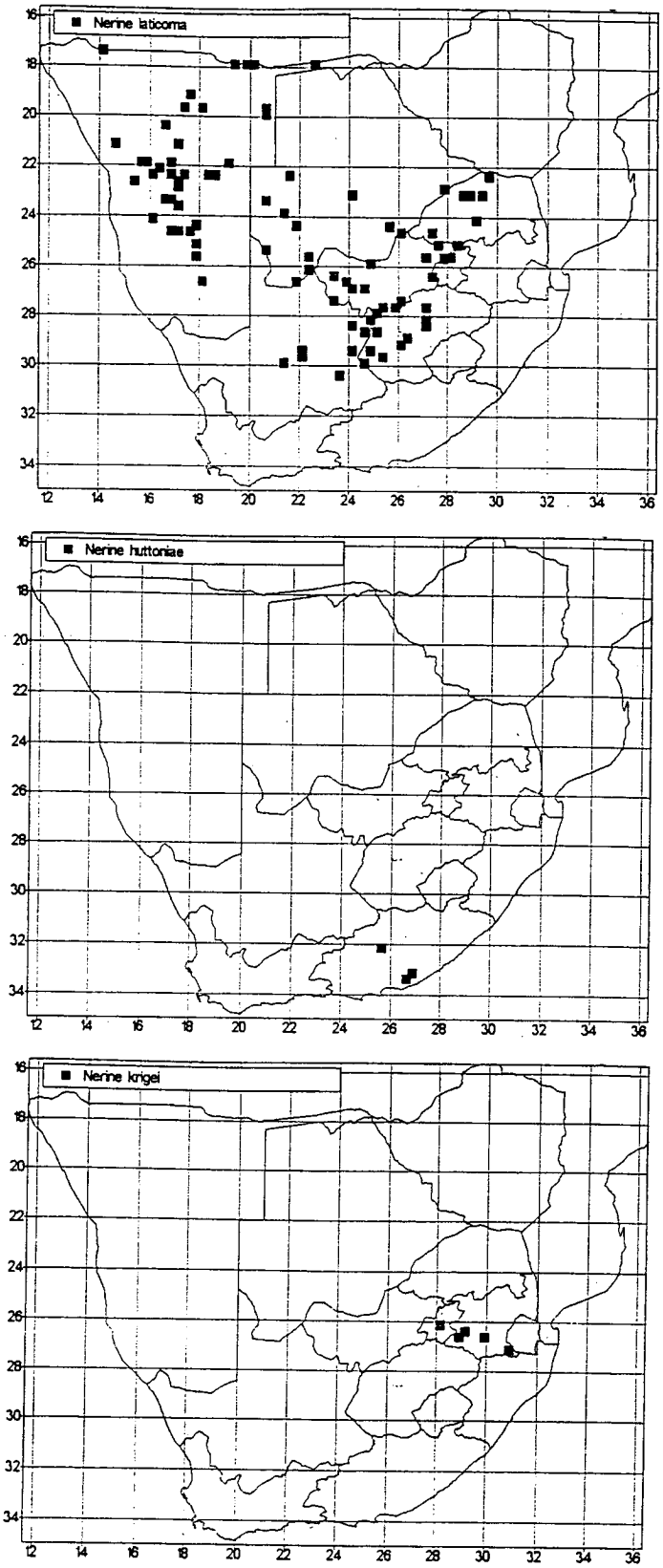

Figure A.1.2 (b-d): Geographical distribution of *Nerine* species: Section Laticomae (ex Traub, 1967) (b) *N. laticomae*; (c) *N. huttoniae*; (d) *N. krigei.*

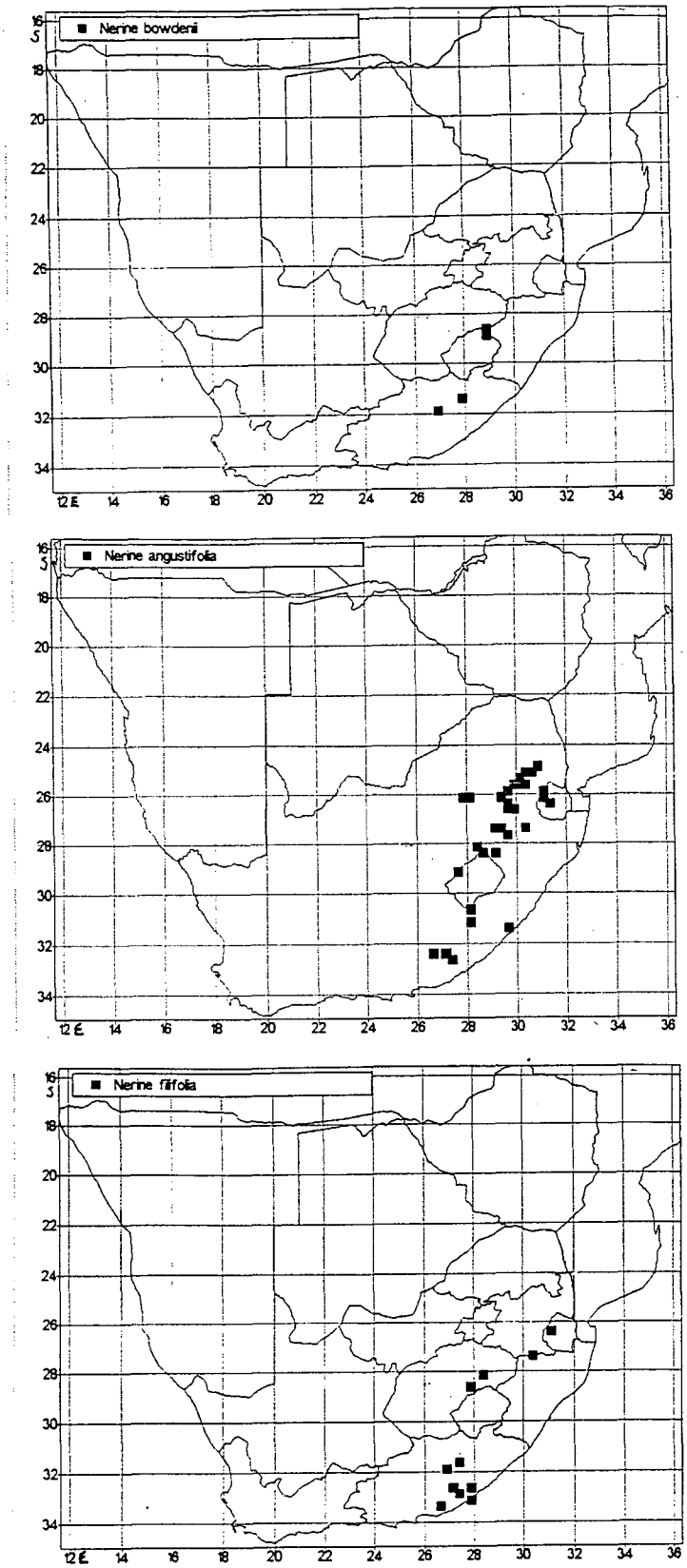

Figure A.1.2 (e-g): Geographical distribution of *Nerine* species: Section Bowdeniae (ex Traub, 1967) (e) *N.bowdenii*; (c) *N. angusifolia*; (d) *N. filifolia.*

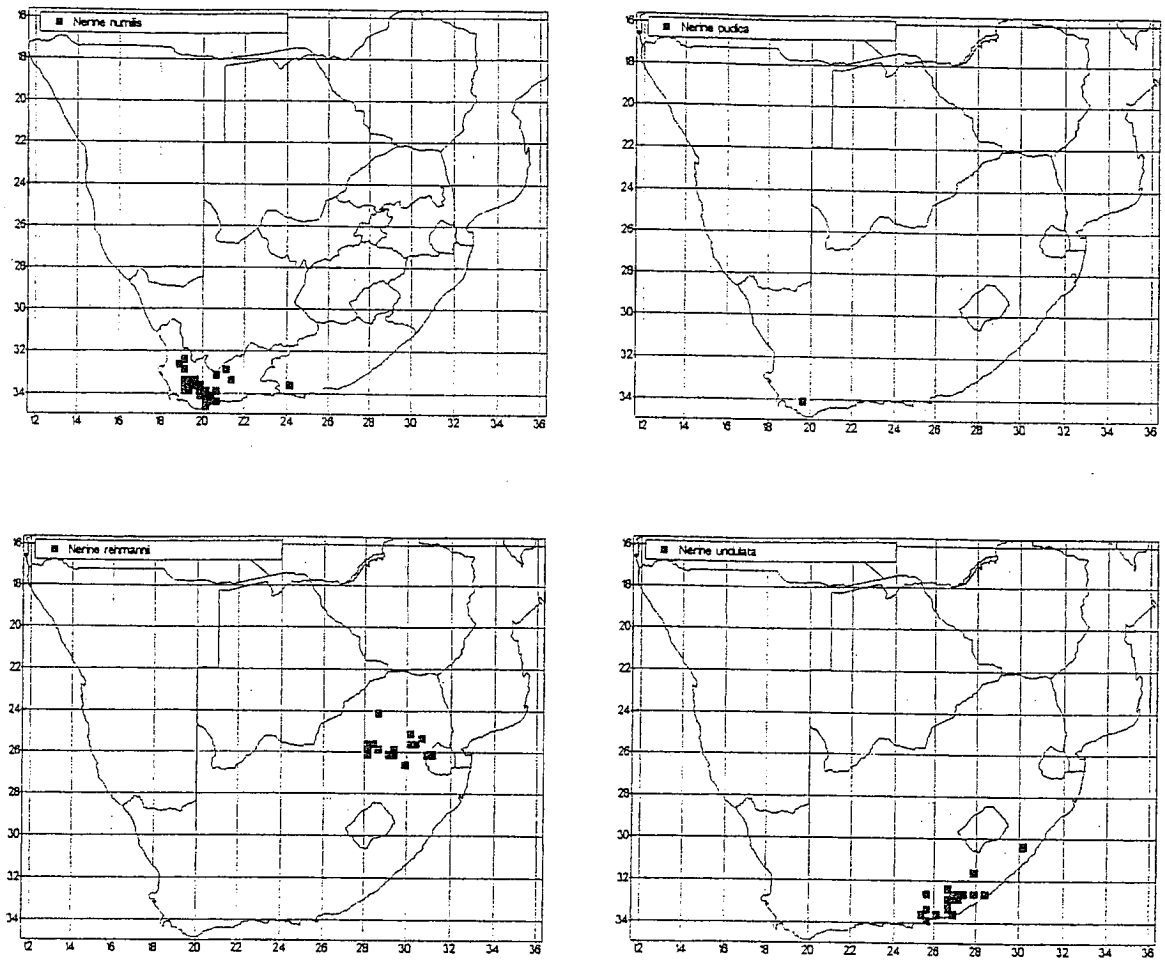

Figure A.1.2 (h-k): Geographical distribution of *Nerine* species: Section Bowdeniae (continued) (h) *N. humilis*; (i) *N. pudica*; (j) *N.rehmanii*; (k) *N. undulata.*

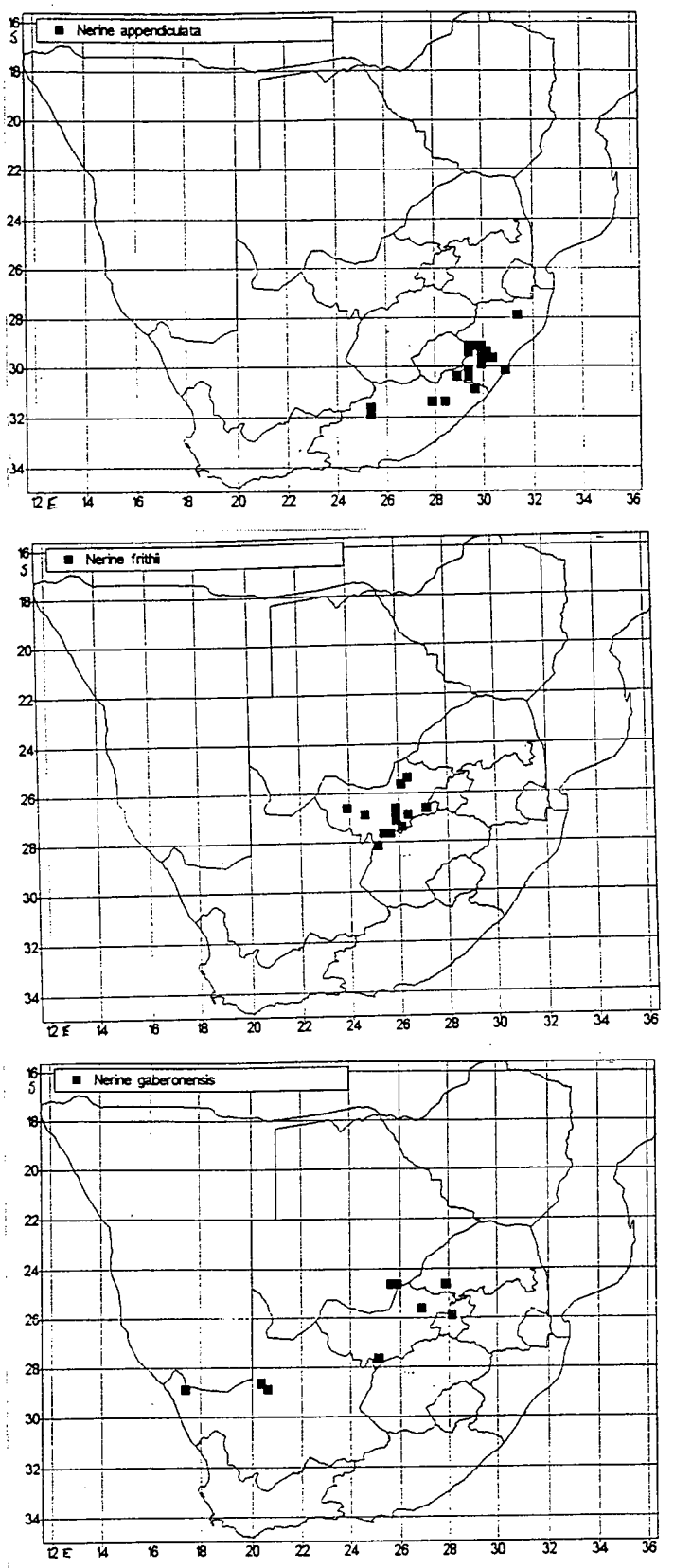

Figure A.1.2 (l-n): Geographical distribution of *Nerine* species: Section Appendiculatae (ex Traub, 1967) (l) *N. appendiculata*; (m) *N. frithii*; (n) *N. gaberonensis*.

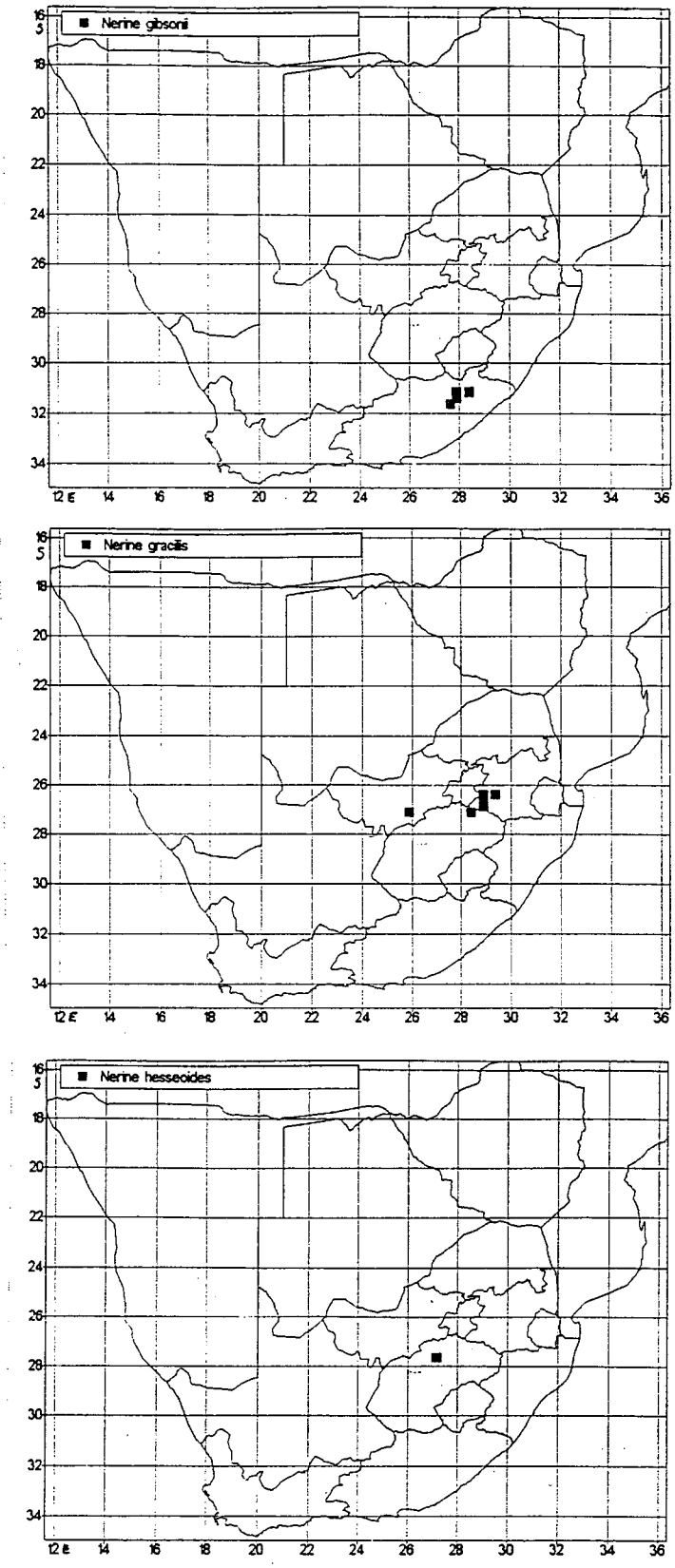

Figure A.1.2 (o-q): Geographical distribution of *Nerine* species: Section Appendiculatae (continued) (o) *N. gibsonii*; (p) *N. gracilis*; (q) *N. hesseoides*.

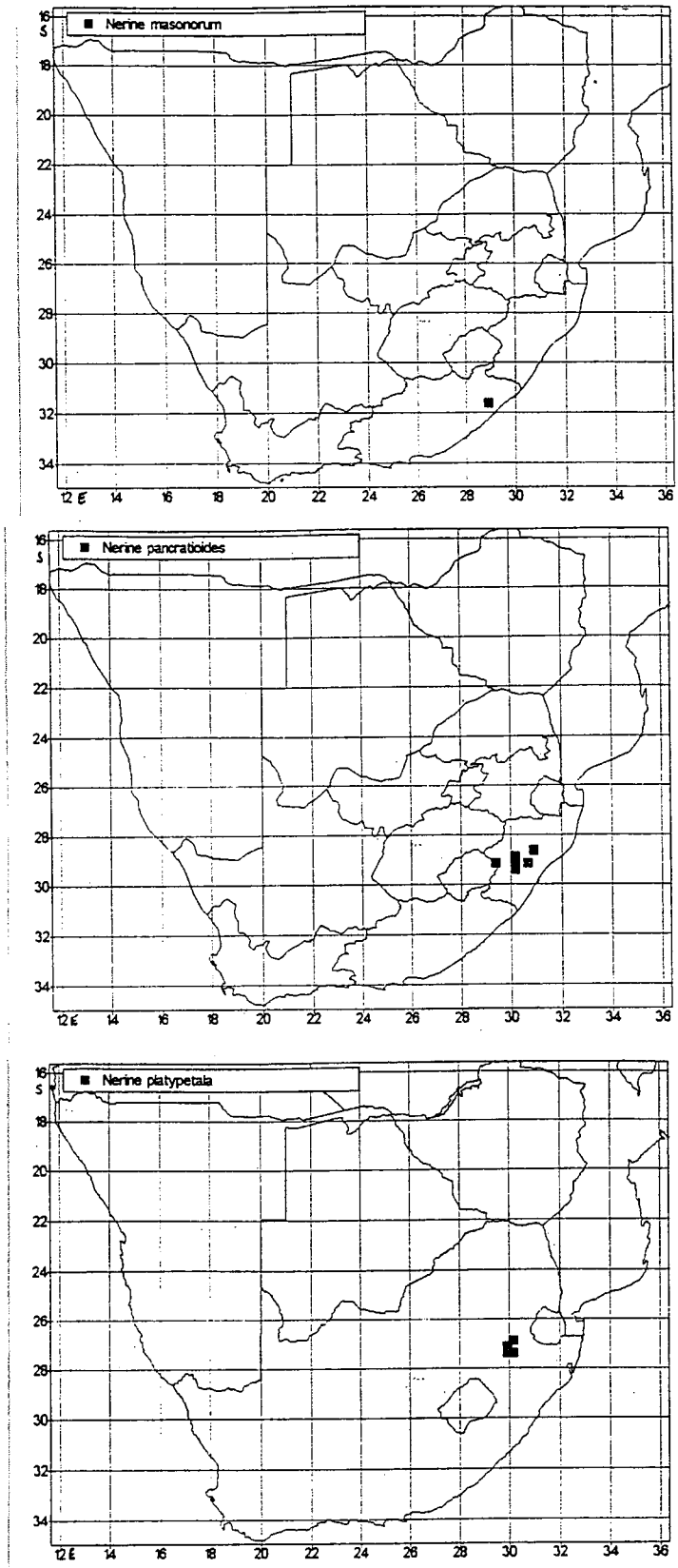

Figure A.1.2 (r-t): Geographical distribution of *Nerine* species: Section Appendiculatae (continued) (r) *N. masonorum*; (s) *N. pancratoides*; (t) *N. platypetala.*

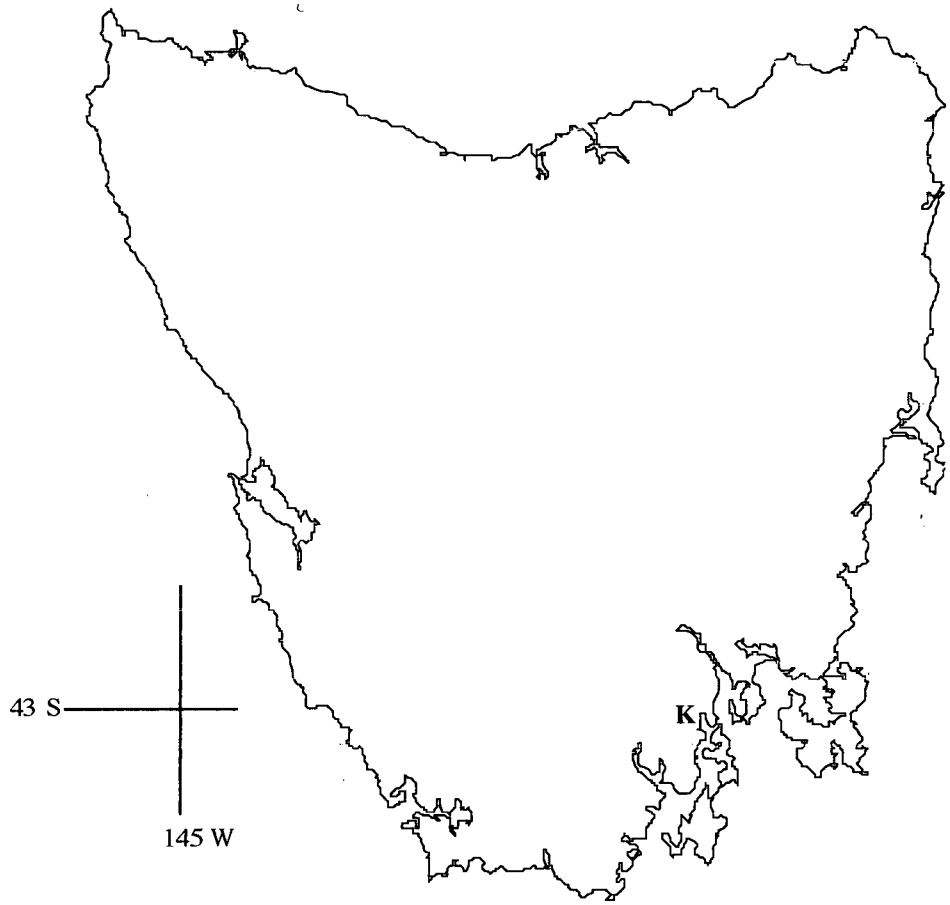

Figure A1.3: Geographical position of Kettering (K), Southern Tasmania, Australia.

Chapter Two

The annual growth cycle, floral development and gamete production in *Nerine*.

2.1 Introduction

Plants of the genus *Nerine* are bulbous perennials native to Southern Africa. Members of the genus exhibit one of three basic growth strategies (Norris, 1974): (i) summer dormancy, where leaves emerge after, or concurrent with, flowering and persist over winter; (ii) winter dormancy, where leaves emerge in spring and die down after autumn flowering; or (iii) evergreen habit, with leaves present throughout the year. Of these, winter dormancy is the most common (Table 1.3). As with other geophytes, production of storage reserves occurs during the active growth period; these reserves can be used to ensure survival during adverse climatic conditions. Although no above ground leaves are present during dormancy periods, the apex remains active and can initiate leaf or floral primordia (Rees, 1989; Theron and Jacobs, 1994; Vishnevetsky *et al.*, 1997).

In *Nerine sarniensis*, autumn flowering is considered to represent the start of a new growing season (van Brenk and Benschop, 1993), whereas in *N. bowdenii*, also autumn flowering, a dormancy period occurs when the leaves senesce at or after flowering, at the beginning of winter (Systema, 1975). In these two species, previous studies have shown that one growth cycle, consisting of development of a series of leaf primordia, followed by initiation of an inflorescence bud, occurs each year (Rees, 1985; van Brenk and Benschop, 1993). The number of leaves between inflorescence buds varies between species and cultivars, with 6-8 being usual (Fortanier *et al.*, 1979). The evergreen species *N. flexuosa* differs in its growth strategy with the number of leaves between inflorescence buds varying from 3-15 and initiation of buds reported to occur more frequently than once per year (Fortanier *et al.*, 1979).

An unusual feature of *Nerine* is the presence of several inflorescence buds, at different stages of development, in the bulb at any one time. In *N. bowdenii* and *N. sarniensis*, these buds are initiated one year apart. After initiation, the buds require over two years of growth and development before flowering occurs (Systema, 1982; Theron and Jacobs, 1994; Vishnevetsky *et al.*, 1997). This phenomenon of preformation has also been reported in another genus of the Amaryllidaceae, *Hippeastrum*, where the time from flower initiation to maturity is 18-24 months (Grainger, 1938). It is yet to be determined how extensive preformation is within the family, but extended bud development occurs in *Amaryllis* (12-13 months [Hartsema and Leupen, 1942; cited in Theron and Jacobs, 1994]) and *Cyrtanthus* (Slabbert, 1997).

The development process has been described in *N. bowdenii* (Theron and Jacobs, 1994) and in *N. sarniensis* x 'Autumn glory' (Vishnevetsky *et al.*, 1997). The current study extends their work and identifies the timing of gametogenesis in several *Nerine* varieties, which has received scant attention to date. Megagametogenesis is of the Polygonum type (Amico and Steffanizzi, 1980), which is commonly found in the Amaryllidaceae (Maheshwari, 1950).

2.2 Materials and methods

2.2.1 Morphology

Bulbs were peeled back to determine diameter at first flowering. This method was also used to identify aborted inflorescences. Number of leaves per growth cycle were counted between inflorescence buds. Mass (fresh weight) of bulbs was determined when in their dormant state, with no above ground leaves present.

Bulb unit terminology used in this chapter adheres to that of Theron and Jacobs (1994) where each growth unit is represented by an inflorescence bud and a series of leaves. A bud due to flower in the current year is designated as 'n', a bud due to flower the following year as 'n+1', and the subsequent year as 'n+2'.

2.2.2 Inflorescence development

Bulbs of *N. sarniensis* x 'Fothergillii major' and *N. bowdenii* x 'Clone 63' were examined at monthly intervals, for two seasons, to observe initiation and development of inflorescence buds. *N. sarniensis* x 'Rosea'[1] was examined at bud initiation, nine months post initiation and monthly in the eight months prior to flowering. Tepal length ten days post anthesis was measured in *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea'. Fresh inflorescences were examined using a Zeiss Stemi SV stereo microscope and an Electroscan 2020 cold stage environmental scanning electron microscope (ESEM). No pre-treatment was required.

2.2.3 Gametogenesis

In the four months preceding flowering, bulbs of *N. bowdenii* x 'Clone 63', *N. flexuosa* 'alba', and *N. sarniensis* x 'Rosea' were examined to identify timing of gametogenesis. Buds were excised and prepared for sectioning by fixation in 4% paraformaldehyde, dehydrated through an ethanol series (O'Brien and McCully, 1981) and embedded in butyl methyl methacrylate (adapted from Webb and Gunning, 1990) or glycol methacrylate (Historesin ®, Jung) (adapted from Wikely and Goodsell, 1994). Sections of 2µm were obtained using a Microm 340E rotary microtome and dried onto slides precoated with 3-aminopropyltriethoxysilane (Sigma). Sections were stained with toluidine blue or the DNA fluorochrome Hoechst 33258 (Sigma) and examined using a Zeiss Axiovert 35 inverted microscope fitted with an HBO mercury vapour lamp. An excitation filter BP365/12, dichroic beam splitter FT395 and barrier LP 397 were used for observation of fluorescence. Fluorescence micrographs were taken using Kodak Ektachrome Daylight 200 film. All other photographs were taken with Kodak Ektachrome 64T Tungsten film.

Microgametogenesis was also observed by anther squashes. Preparations were unstained or stained with toluidine blue (viewed by light microscopy) or Hoechst 33258 (viewed under fluorescence). Microtubules were detected by immunofluorescence microscopy. Anther squashes were treated with anti-ß tubulin and SAM-FITC antibodies (adapted from Fowke *et al.*, 1984; Webb and Gunning, 1990; see Appendix C.1), and viewed under fluorescent light, as described above.

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[1] The cultivar *N. sarniensis* x 'Rosea' (also referred to as x 'Roseum') is an hybrid of Australian origin, and differs from the *N. sarniensis* var 'Rosea' of Herbert. For further discussion see Chapter 10.

2.3 Results

2.3.1 Morphology

Bulbs consist of circular scales, which represent the bases of the green leaves. The outer 3-4 papery, brown and desiccated layers give way to fleshy scales and finally thinner bases with expanding leaves. Inflorescences, when present, form between two non-circular scales (Plate 2.1 a). Residual flower stalks can be found in outer bulb scales, and these together with the outer scale leaves gradually desiccate and slough off. The minimum bulb diameter for first flowering in *Nerine* varies within and between species (Table 2.1), and usually represents 4-5 seasons' growth. *Nerine* bulbs must achieve a minimum size before an inflorescence bud is initiated, and two further growing seasons are required before the bud develops to flowering size. This size (recorded as diameter and bulb mass in Table 2.1) is a product of number of growing seasons and the number of leaves produced each season. The number of leaves per growing season varies within and between species, ranging from 3-8 (Table 2.1).

Cultivar	Bulb mass (g)	Diameter (mm)	Leaves per growth cycle
N. bowdenii x 'Clone 63'	34	51±2.0	6.3±0.2 (5-8)
N. flexuosa 'alba'	38	-	5.5±0.5 (5-8)
N. sarniensis x 'Fothergillii major'	45	54±2.0	6.2±0.3 (4-8)
N. sarniensis x 'Rosea'	12	-	4.5±0.2 (3-8)

Table 2.1: Mass and diameter of (dormant) bulb at first flowering, and number of leaves present between successive inflorescence buds (range shown in parentheses).

2.3.2 Inflorescence development

A mature bulb of *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Fothergillii major' will contain up to three inflorescence buds at any one time (Plates 2.1 b, 2.1 c). Both *N. bowdenii* and *N. sarniensis* exhibit an annual growth cycle during in which typically only one bud is initiated. Timing of inflorescence initiation is also similar, occurring in spring (Fig. 2.1), and the complete developmental process requires over two years to complete (32 months in *N. bowdenii* x 'Clone 63' and 29 months in *N. sarniensis* x 'Fothergillii major').

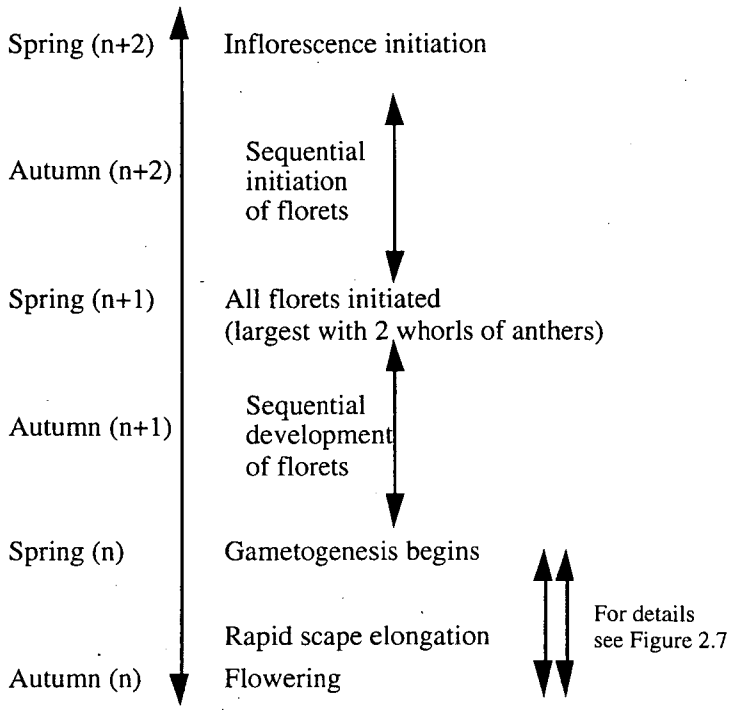

Figure 2.1: General development timetable for inflorescence of *Nerine*.

In *N. bowdenii*, a new inflorescence is initiated mid-spring (Plate 2.1d), and two florets in the early stage of development are present by early summer. Initiation of a new inflorescence in *N. sarniensis* x 'Fothergillii major' occurs in late spring, with floral primordia being evident by mid-summer and floral organs beginning development in autumn. In both species, florets are initiated sequentially, and within each floret the same pattern of development is seen (*i.e.* two whorls of tepals, first whorl of anthers, second whorl of anthers and tri-carpellate gynoecium [Plates 2.1e-h, 2.2 a]). However, distinct patterns of growth could be identified for the two species.

During the first season of growth in *N. bowdenii* x 'Clone 63', the length of the inflorescence increases gradually and reaches a length of approximately 3.5mm (period of elongation is designated I in Figure 2.2). After this, a relatively constant length is maintained with this non-elongation period encompassing the bulb's dormancy period from May-August (late Autumn-Winter). During this first growth season, florets are initiated one at a time until the final number (usually 4-8) are formed. Development of individual florets proceeds slowly within the bud, continuing over the period when the bud is not elongating. Floret development is not uniform with florets of different stages present in the same bud (Plate 2.2b). In

winter, at the end of this first season, the largest florets within the inflorescence have two whorls of anthers while the smallest florets have tepals only.

The beginning of the second annual growth cycle is marked by a gradual increase in bud length in *N. bowdenii* (designated as II in Figure 2.2). Again, this is followed by a suspension of elongation during the dormancy period. Floral organ development continues, occurring in each floret sequentially and continues through the dormancy period. In autumn, one year prior to flowering, the largest florets have the tri-carpellate gynoecium formed and elongating but not yet fused. The smallest florets have three anthers (Plate 2.1f-g), with the majority of florets having both whorls formed. The fusion of the gynoecium is evident in the largest florets from mid-spring with ovules forming in late spring. Gynoecial fusion in smaller florets is completed by mid-summer with ovules forming, after fusion and elongation, in late summer. At the stage of gynoecial fusion, anthers appear to be well developed, however filaments have yet to elongate (Plate 2.2c).

A sharp increase in the inflorescence length of *N. bowdenii* occurs in mid-spring, two years after initiation (designated as III in Figure 2.2). Initially this is due to spathe elongation that slows by early summer with spathe length remaining approximately the same for the next three months. Increase in bud length after this time is due mainly to scape elongation, which begins in early summer, when it accounts for over 30% of total bud length. This increases to over 50% during January-February (late summer). Rapid scape elongation occurs during March-April (early - mid autumn), immediately prior to flowering, and is accompanied by a further elongation of spathe length, as well as the appearance of pigment in the inflorescence. Elongation of individual florets continues after the spathe valve has opened (Plate 2.2d), and proceeds rapidly prior to opening of the individual floret (Fig. 2.3). Tepals do not reach maximum length until approximately five days after anthesis of the floret.

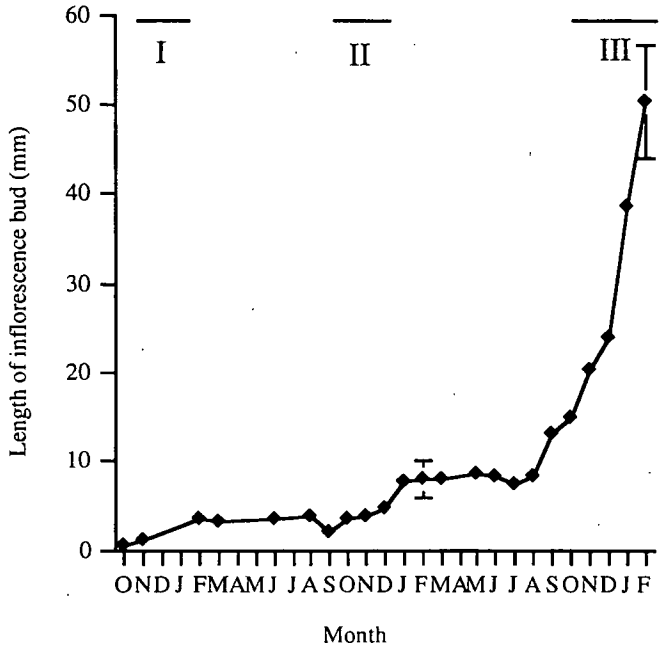

Figure 2.2: Growth of inflorescence bud in *N. bowdenii* x 'Clone 63'. I, II and III represent periods of inflorescence elongation.

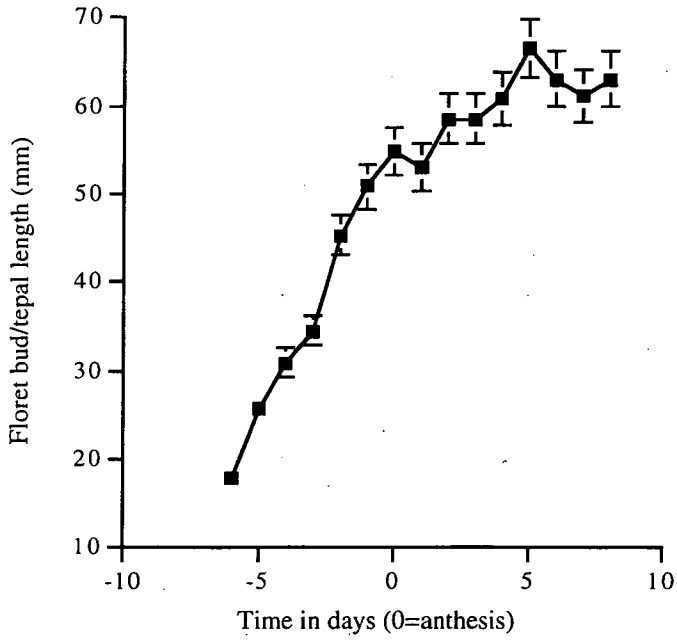

Figure 2.3: Growth of individual floret buds in *N. bowdenii* x 'Clone 63' after opening of the spathe valve.

In *N. sarniensis*, growth of the inflorescence is relatively steady for the first two growing seasons with elongation not punctuated by periods of dormancy (Figs 2.4, 2.5). In this respect, it contrasts with the growth pattern of *N. bowdenii*. In *N. sarniensis* x 'Fothergillii major' there is a steady increase in bud length in the first growing season during which florets are initiated sequentially (Fig. 2.4). The final number of florets varies from 8-15. By the end of this first growing season, the perianth and the first whorl of anthers are formed in all florets. The increase in bud length continues at approximately the same rate during the second growing season (Fig. 2.4). The period of rapid spathe elongation begins in late spring, four months prior to flowering in early autumn, with rapid elongation of the scape beginning in summer and accelerating prior to flowering in early autumn (Fig. 2.4). A similar pattern of gradual elongation is found in *N. sarniensis* x 'Rosea', however the final elongation phase begins earlier, in winter, which corresponds with the earlier flowering period of this cultivar (Fig. 2.5). As in *N. bowdenii*, elongation of individual florets continues after the spathe valve has opened, however, tepals reach maximum length in *N. sarniensis* x 'Rosea' approximately two days after anthesis of the individual floret (Fig. 2.6).

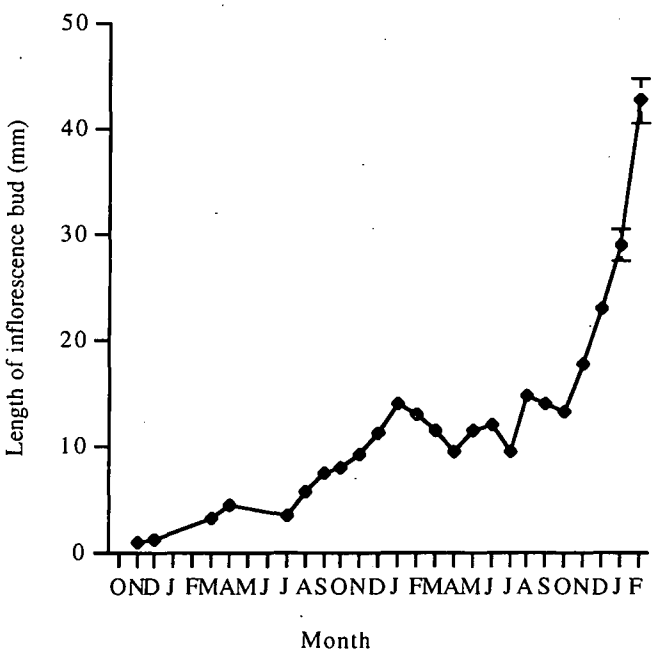

Figure 2.4: Growth of inflorescence bud of *N. sarniensis* x 'Fothergillii major'.

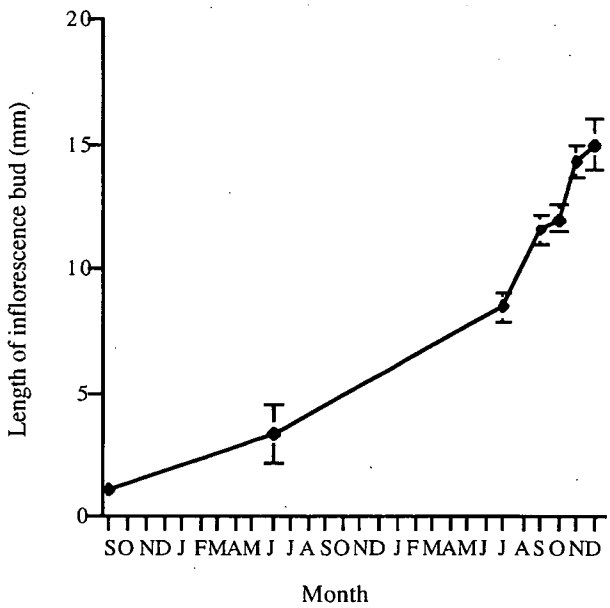

Figure 2.5: Growth of inflorescence bud of *N. sarniensis* x 'Rosea'.

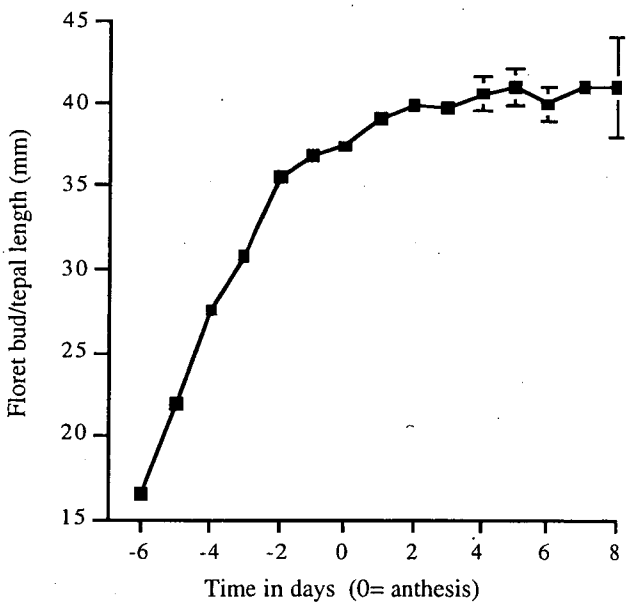

Figure 2.6: Growth of individual floret buds in *N. sarniensis* x 'Rosea' after opening of the spathe valve.

In *N. sarniensis*, floret development within the inflorescence is asynchronous, as seen in *N. bowdenii*. Consequently, florets can be found at different developmental stages depending upon position in the sequence of initiation. Also, the pattern of organ development in *N. sarniensis* is sequential and similar to *N. bowdenii*. In winter, at the end of the first season, the first whorl of anthers is present in the largest

florets. The initiation of the second whorl of anthers in largest florets occurs in spring of the second season. This is followed by the development of the unfused tri-carpellate gynoecium, which occurs progressively in florets over the second season. Fusion of the gynoecium occurs prior to ovule development, at least in the largest florets. The ovarian cavity is evident, at the base of the stylar canal, from late spring (Plates 2.2e), with ovules present in early summer. The ovules arise from the outer wall of the locule and the uppermost ovules are the first to develop.

2.3.3 Gametogenesis

In *Nerine*, production of the pollen grain, embryo sac and the gametes occurs relatively late in the development of the inflorescence. In all varieties of *Nerine* studied, microsporogenesis is not synchronised within the bud, occurring first in larger florets and not occurring in smaller florets until up to three months later. However, binucleate pollen grains are present in both whorls of anthers at anthesis. This asynchrony is also true of embryo sac development. Mature embryo sacs are present at anthesis in all the cultivars studied (*N. bowdenii* x 'Clone 63', *N. sarniensis* x 'Fothergillii major', *N. sarniensis* x 'Jill', *N. sarniensis* x 'Rosea' and *N. filamentosa*). The egg apparatus is at the micropylar end, with the egg cell positioned slightly above the two prominent synergids (Plates 2.3a-c).

In *N. bowdenii*, microsporocytes (pollen mother cells [PMCs]) are seen in the first whorl of anthers of the largest florets from early spring (Fig. 2.7; Plates 2.4a-b). Pollen grain meiosis (PGM) occurs in these largest anthers during late spring-early summer (Plates 2.4a-e). Subsequently, microspore tetrads and released microspores are found (Plates 2.4 f-g). Microspore tetrads in the smallest florets are evident by late summer, at which time unicellular pollen grains, with exine, are present in the largest florets. By early autumn, immediately prior to flowering, all pollen grains are formed. Ovules form in early summer in the larger florets, but are not present in smallest florets until three months later. Staggered development also occurs in ovules within a single floret which may be at varying stages of maturity. Embryo sac formation is also asynchronous, with the first embryo sacs seen in mid-summer in the largest florets of *N. bowdenii.*

In *N. sarniensis* x 'Rosea', PMCs are found in the largest florets during spring (Fig. 2.7) and mature pollen grains are present in anthers by mid-summer. In early summer ovules are formed in the larger florets, whilst the gynoecium is yet to fuse in the smaller florets. Ovules are apparent in smaller florets during mid-late summer,

less than one month before flowering. Embryo sac formation occurs first in the largest florets and is completed by anthesis in the smallest florets. Gametogenesis in *N. sarniensis* x 'Fothergillii major' was not followed due to its triploid nature, which may result in atypical gamete formation. However, sectioning mature ovules revealed apparently normal embryo sacs in this cultivar (Plate 2.3 a). Seeds have also resulted from controlled crosses using *N. sarniensis* x 'Fothergillii major' as a seed parent. Similarly, pollen of *N. sarniensis* x 'Fothergillii major' has been found to germinate during *in vitro* trials.

In *N. flexuosa* 'alba', PMCs are found in the largest anthers in early summer, with PGM occurring during the following month and pollen grains present in anthers by late summer (Fig. 2.7). PGM in the smaller florets occurs in late summer, approximately six weeks prior to flowering. Ovule development in the largest florets occurs in mid-summer, but is much later in smaller florets, occurring in the final weeks before flowering. Megagametogenesis in these florets is close to flowering time. Late megagametogenesis is also found in the smaller florets of *N. filamentosa* where embryo sac formation occurs just four days prior to anthesis (Plate 2.5a). At this stage, binucleate pollen grains are found in all anthers (Plates 2.5b-c).

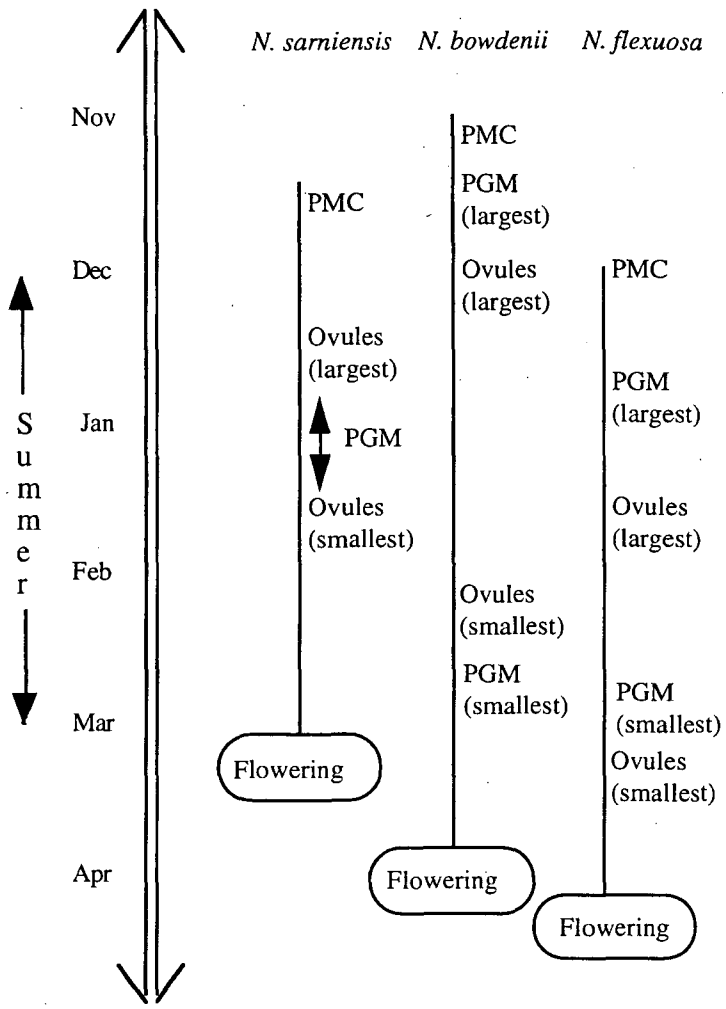

Figure 2.7: Development timetable of *Nerine* species. PMC = pollen mother cells; PGM = pollen grain meiosis; Largest/Smallest refers to size of floret relative to others in the inflorescence.

2.4 Discussion

Necessary attainment of a minium bulb size before flowering in *Nerine* is due to: (i) the bulb needing to acquire a certain size before initiating an inflorescence; and (ii) the lengthy period required for the inflorescence to reach maturity (over two growing seasons). In *N. bowdenii* and *N. sarniensis*, these factors could not be achieved in under four growing seasons (the length of this study). A generation time of at least three years has been reported in *N. platypetala* (Craib, 1996) and *N. masonorum* (Koopowitz, 1986). A long generation time is characteristic of many members of the

family (see Appendix A.2.1), which has implications for conservation (Koopowitz, 1986) and plant breeding.

The flowering diameter of the bulb is a product of the number of growing seasons and the number of leaves grown in each cycle. In *N. bowdenii* and *N. sarniensis*, this had been reported to be 12-14cm (Rees, 1979). However, the cultivars of these species used in the current study flowered at a much smaller diameter (5-6cm). The average number of leaves produced per growth cycle was similar in both cultivars. *N. sarniensis* x 'Rosea', a cultivar with smaller bulbs, had a lesser flowering diameter with fewer leaves produced each growth cycle than the larger *N. sarniensis* x 'Fothergillii major'. Nevertheless, seedlings of *N. sarniensis* x 'Rosea' did not flower until at least four seasons' growth was completed. Thus the number of leaves produced each growth cycle was found to vary within as well as between species, which agrees with previous studies (Rees, 1985; van Brenk and Benschop, 1993). Variation between bulbs of a single cultivar is also found and is likely to be due, at least in part, to environmental factors as this has been shown to influence the number of florets in an inflorescence in *Nerine* (see Chapter 7).

In *N. bowdenii*, the observed 32 month period from initiation to flowering is longer than the 28 months reported for the same species by Theron and Jacobs (1994). This could be due to the differing environmental conditions for the two studies and/or a variation in the cultivars used. The 29 month cycle observed in *N. sarniensis* x 'Fothergillii major' is considerably longer than those reported for *N. sarniensis* cultivars by Vishnevetsky and co-workers (1997) (22 months) and Rees (1979) (over 18 months, but prior to initiation of a third inflorescence). Again, climatic conditions, as well as cultivar variation, may account for this discrepancy. Certainly, it is consistent with the longer period found in *N. bowdenii*, suggesting the Tasmanian climate may be influencing the growth cycles. The scenario of climatic conditions influencing growth phenology and flowering time in *N. bowdenii* is also supported by the work of Shillo and co-workers (1997).

In *Nerine*, floret development occurs in three phases outlined by Theron and Jacobs (1994): (i) floral initiation; (ii) floral development; and (iii) floral elongation. However, in *N. bowdenii*, actual increases in inflorescence bud length occur in three separate phases punctuated by two non-elongation periods that correspond to the periods of vegetative dormancy. This cessation of bud elongation during the dormancy periods corresponds with the observations of Systema (1975), who

reported floral buds to be dormant as soon as the old leaves die at the beginning of winter. However, whilst elongation of the entire inflorescence does not occur during this period, differentiation of floral organs is occurring. The first gradual elongation phase coincides with the initiation of the first floret with the second elongation phase occurring during differentiation. The final elongation phase coincides with leaf growth prior to flowering and is longer than the four month period reported by Theron and Jacobs (1994).

Unlike *N. bowdenii*, inflorescence bud development in *N. sarniensis* appears to be independent of dormancy, as demarcated by absence of above ground leaves. It proceeds through three distinct phases: (i) the first year, where florets are initiated and bud length increases gradually; (ii) the second year, where floral organs are developing, with only a gradual increase in overall size; and (iii) the final 4-5 months, marked by spathe and scape elongation prior to anthesis. Elongation of the inflorescence occurs during all three dormancy periods, with the greatest elongation occurring in the period of dormancy immediately prior to flowering.

The pattern of organ formation in *Nerine*, with whorls of similar organs appearing simultaneously within a floret is a common pattern. The development of the androecium preceding that of the gynoecium is also commonly found (Greyson, 1994). The development of three separate carpels, followed by fusion relatively late in the development process, is also seen in *Allium* (Esau, 1965). Given the usual pattern in which androecial development precedes gynoecial, it would be expected that *Nerine* pollen grain development occurs prior to embryo sac development. This development sequence has also been found in a number of genera including *Hordeum*, *Oryza* and *Nicotiana* (Yang and Zhou, 1982).

In *Nerine*, production of the gametophytes (the pollen grain and embryo sac), occurs relatively late in the development sequence of the inflorescence. Little previous work has been completed on the timing of gametogenesis in this genus. Richards (1990; cited in Theron and Jacobs, 1994) reported that PGM occurs during the four month scape elongation period. This study found that PGM did occur during the final elongation period, but began earlier than four months prior to flowering, at least in the largest florets of *N. bowdenii*. Importantly, it has been established that PGM is not synchronised in the bud, with anthers in smaller florets undergoing PGM up to two months after those in larger florets.

Ovule development as well as subsequent megasporogenesis and megagametogenesis are also not synchronised within an inflorescence. Again these processes are sequential, occurring first in the larger florets and progressing to the smaller florets over a period of three months. Vishnevetsky and co-workers (1997) found ovules in *N. sarniensis* in early summer, which corresponds to these results for the larger florets. Megagametogenesis in *Nerine* has been described by Amico and Steffanizzi (1980), and is of the Polygonum type, commonly found in the Amaryllidaceae (Maheshwari, 1950). Although it appears mature embryo sacs are present in *Nerine* ovules by anthesis of an individual floret, late formation in the week prior to anthesis has been found in smaller florets.

Inflorescence preformation found in *Nerine* appears to be of some adaptive significance, as it operates in many, if not all, members of the genus. It is found in at least one other genus in the family (*Hippeastrum* [Grainger, 1938]), and other closely related genera have species exhibiting extended bud development (Theron and Jacobs, 1994). Organ preformation is reportedly common in arctic and alpine plants, where it can be crucial to their survival (Diggle, 1997), allowing rapid development and maturation of the reproductive organs, as well as seed production in a limited growing season. As the distribution of both *Nerine* and *Hippeastrum* is generally sub-tropical (although some species of *Nerine* grow in montane districts, subject to freezing in winter), it would initially appear to be contradictory to this model. Nevertheless, the concept of a limited growing season may well apply to many species of *Nerine,* at least those that exhibit a period of dormancy.

Vegetative dormancy is a mechanism for survival through adverse climatic periods (Rees, 1989) and the growth strategies of either summer and winter dormancy suggests harsh conditions during these seasons. For *Nerine* species, these harsh conditions are probably related to the limited rainfall seasons. Therefore the ability of inflorescences to rapidly elongate and proceed through the reproductive process in a short period of time, may be advantageous under these conditions. This is consistent with the summer dormancy exhibited by *Nerine* species indigenous to areas of winter rainfall (southern regions) and winter dormancy of species from northern regions, with rainfall predominantly in the summer. Interestingly, species occurring between these geographical extremes, such as *N. flexuosa* have no distinct dormancy period (Warrington *et al.*, 1989) and will remain evergreen in cultivation with continued watering (Norris, 1974). The implications of preformation, with particular regard to horticultural applications, are discussed in Chapter 3.

2.5 Conclusion

In *Nerine*, the inflorescence is preformed within the bulb scales, with development extending up to 32 months prior to flowering. At any one time, a bulb of *N. bowdenii* or *N. sarniensis* may contain up to three inflorescences, each having been initiated one year apart. In Tasmania, inflorescence initiation is in mid-spring, after which individual florets are initiated sequentially. Development of floral organs within florets is continuous. In *N. bowdenii*, however, increase in bud length is not continuous, with growth ceasing during periods of vegetative dormancy.

Gametogenesis occurs relatively late in the development sequence, four to five months prior to flowering. As with floret development, this is asynchronous and occurs over a period of up to five months.

Plate 2.1a *In situ* inflorescence of *N. bowdenii* x 'Clone 63'. Scale bar = 0.5cm.

Plate 2.1b n+1(left) and n+2 inflorescence of *N. bowdenii* x 'Clone 63' with spathe intact. Scale bar = 0.5cm.

Plate 2.1c n+1(left) and n+2 inflorescence of *N. bowdenii* x 'Clone 63' with spathe removed. Scale bar = 0.5cm.

Plate 2.1d Floral primordia of n+2 inflorescence of *N. bowdenii* x 'Clone 63'. Scale bar = 0.2mm.

Plate 2.1e Longitudinal section of *N. bowdenii* x 'Clone 63' floret (n+2) showing tepal primordia. Scale bar = 0.2mm. (Stain: Toluidine blue.)

Plate 2.1f Longitudinal section of *N. bowdenii* x 'Clone 63' floret (n+1), with completely formed tepals (t) and first whorl anther buds (a). Scale bar = 0.5mm. (Stain: Toluidine blue.)

Plate 2.1g n+1 floret of *N. bowdenii* floret with tepals (t) and first whorl of anther buds (a). Scale bar = 1mm.

Plate 2.1h Longitudinal section of *N. bowdenii* x 'Clone 63' floret (n+1), with tepals (t) and both whorls of anthers (a) present.

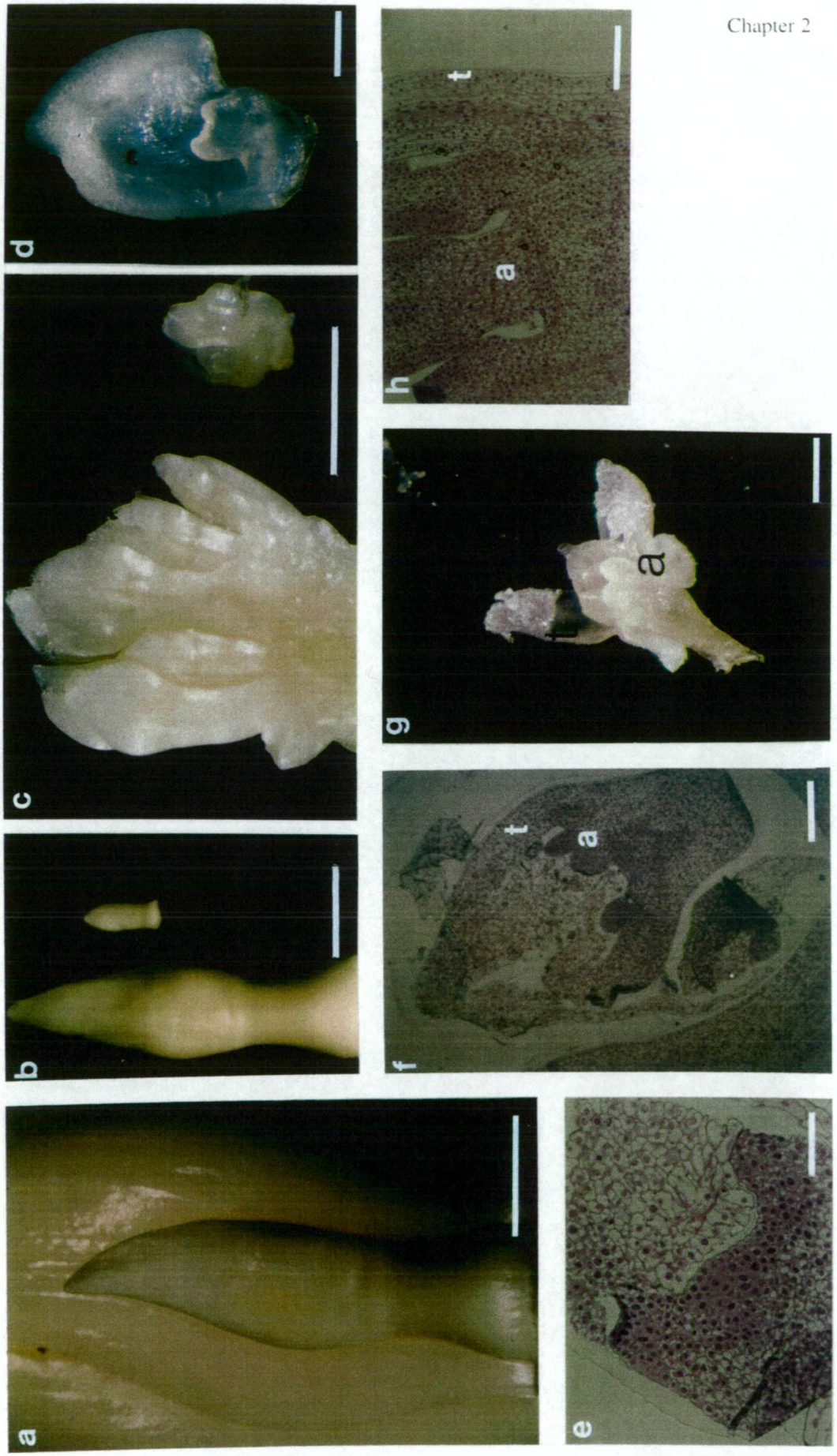
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Plate 2.2a Floret of *N. bowdenii* x 'Clone 63', six months prior to flowering. All whorls have formed, with gynoecium consisting of three carpels not yet fused. There is no evidence of ovary differentiation. (a) = anther, (g) = gynoecium, (t) = tepals. Scale bar = 1mm.

Plate 2.2b n+2 floret of *N. bowdenii* x 'Clone 63' with florets at differing stages of development within the inflorescence. Scale bar = 2mm.

Plate 2.2c Floret of *N. bowdenii* x 'Clone 63', with tepals removed. The gynoecium (g) has fused and ovules (o) are present. Scale bar = 1mm.

Plate 2.2d Spathe of *N. bowdenii* x 'Winter Cheer' opening to reveal the inflorescence. Scale bar = 1cm.

Plate 2.2e Longitudinal section of *N. bowdenii* x 'Clone 63' gynoecium, four months prior to flowering. The stylar canal (s) and locules (l) are present. Scale bar = 0.5mm. (Nuclei are stained by the DNA fluorochrome Hoechst.)

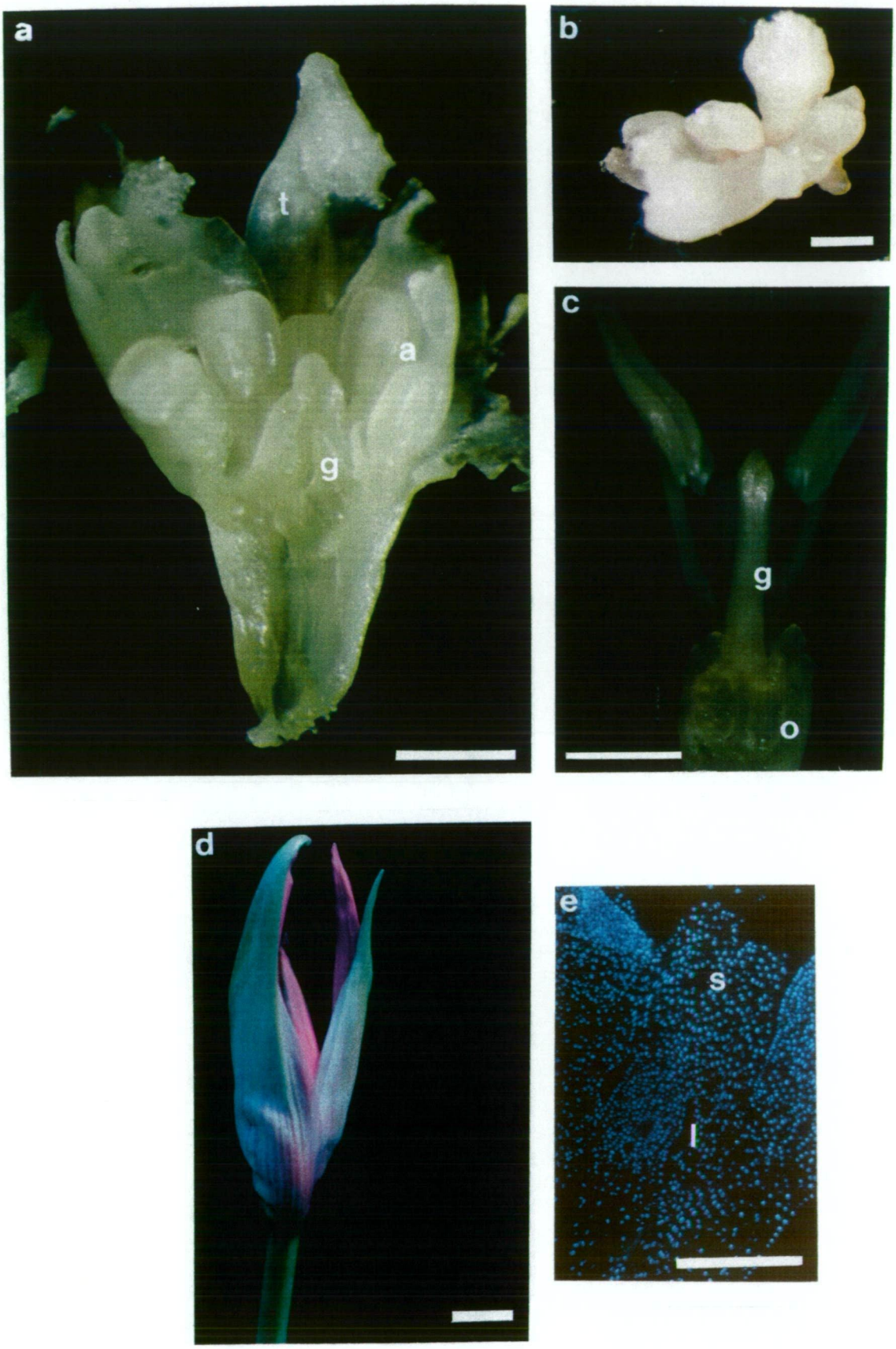
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Plate 2.3a Longitudinal section of *N. sarniensis* x 'Fothergillii major' ovule, through the embryo sac. The synergids (s) with prominent egg apparatus are visible at the micropylar end (m) of the embryo sac. Scale bar = 100μm. (Stain: Toluidine blue.)

Plate 2.3b Longitudinal section of *N. sarniensis* x 'Fothergillii major' embryo sac showing egg cell (e) and two prominent synergids (s). Scale bar = 50μm. (Stain: Toluidine blue.)

Plate 2.3c Longitudinal section of *N. sarniensis* x 'Fothergillii major' embryo sac showing the two polar nuclei of the central cell. Scale bar = 50μm. (Stain: Toluidine blue.)

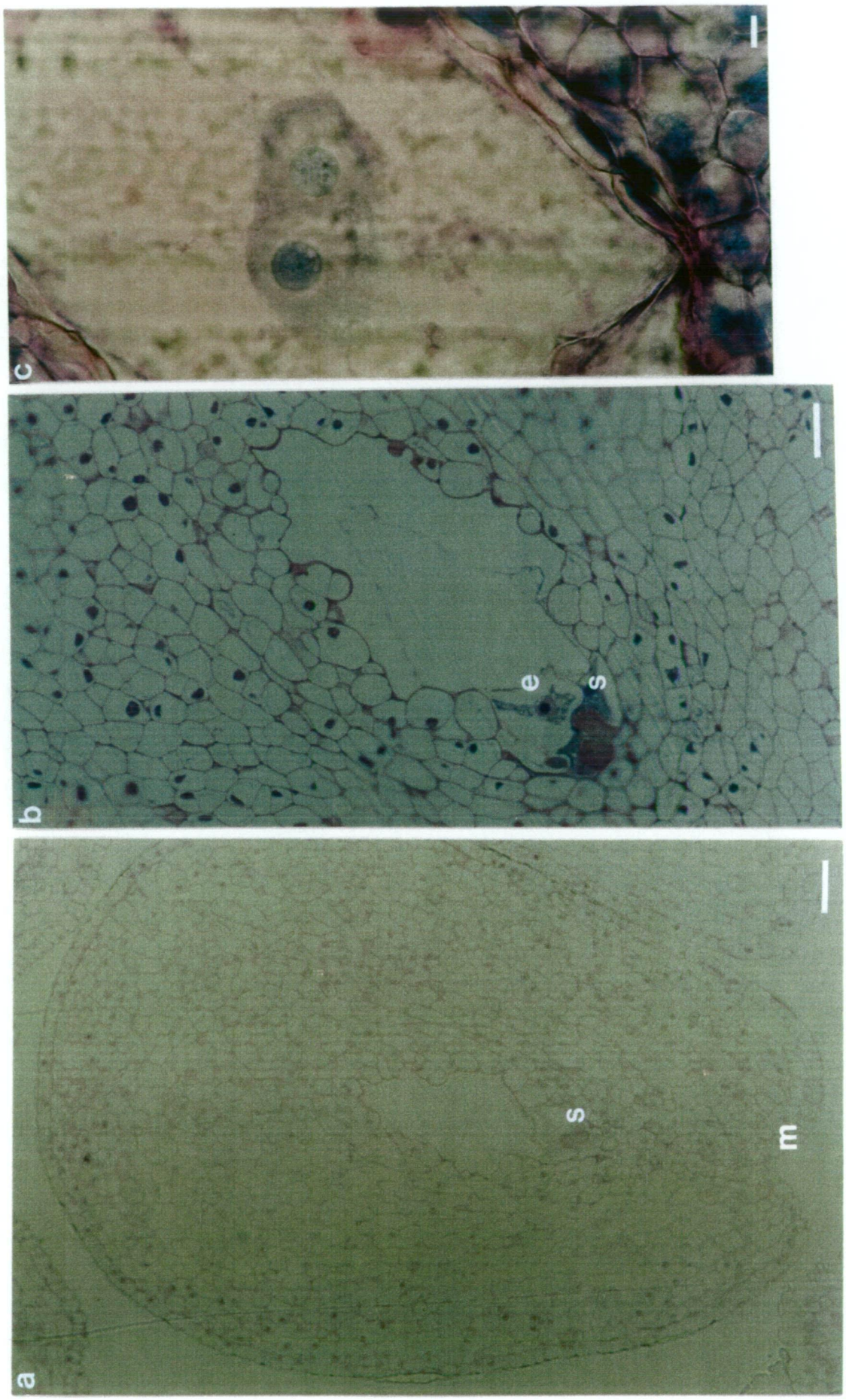
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Plate 2.4a Longitudinal section of *N. bowdenii* x 'Clone 63' anther showing microspore mother cells (m). Scale bar = 100µm. (Stain: Toluidine blue.)

Plate 2.4b Isolated microspore mother cell. Scale bar = 50µm. (Unstained.)

Plate 2.4c Immunofluorescence micrograph showing the microtubular spindle formation during pollen grain meiosis. Scale bar = 100µm.

Plate 2.4d First division during pollen grain meiosis. Scale bar = 100µm. (Stain: Hoechst.)

Plate 2.4e Second division during pollen grain meiosis. Scale bar = 50mm. (Stain: Toluidine blue.)

Plate 2.4f Pollen grain tetrads prior to release from pollen mother cell. Scale bar = 50µm. (Stain: Toluidine blue.)

Plate 2.4g Unicellular microspores. Scale bar = 100µm. (Stain: Toluidine blue.)

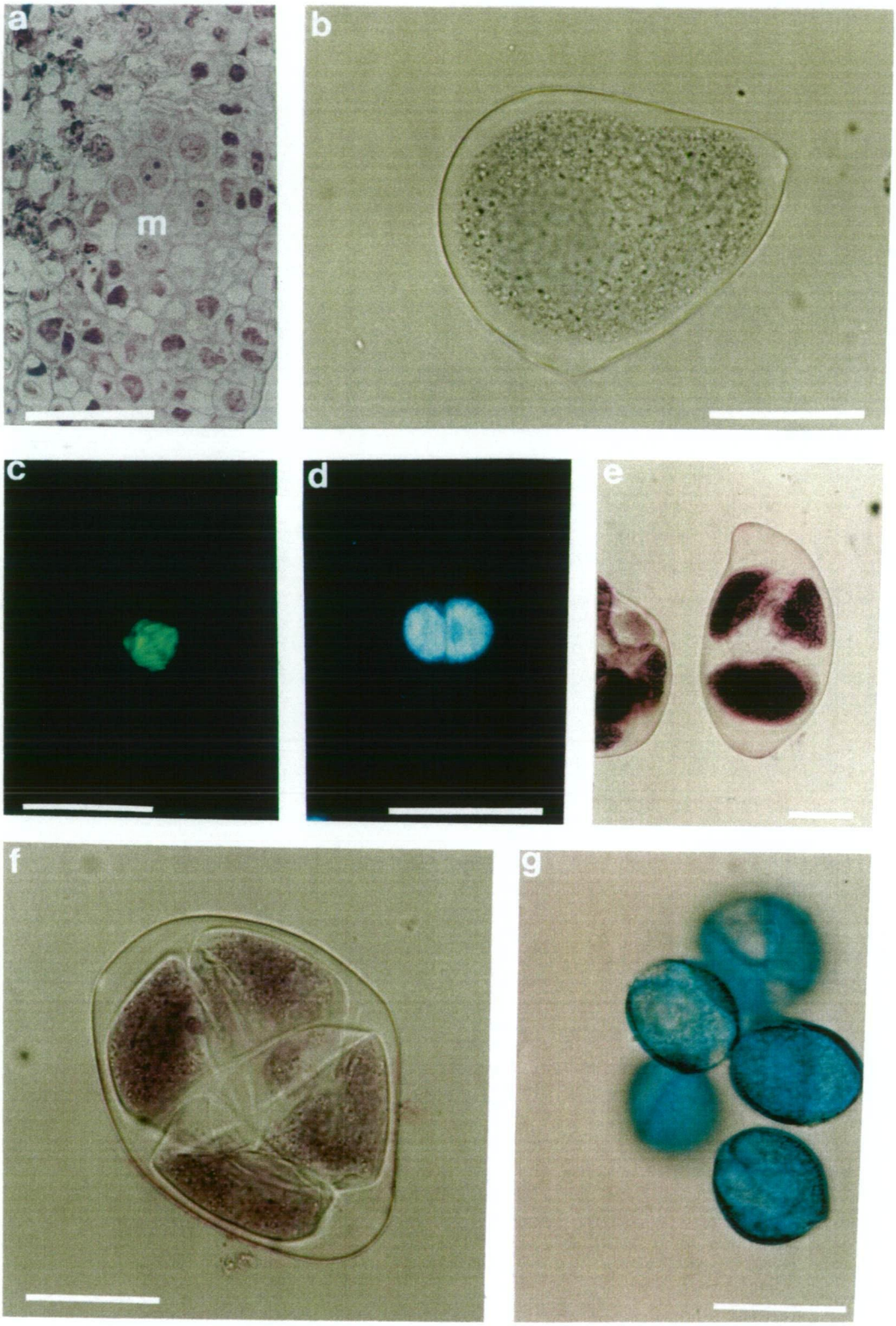
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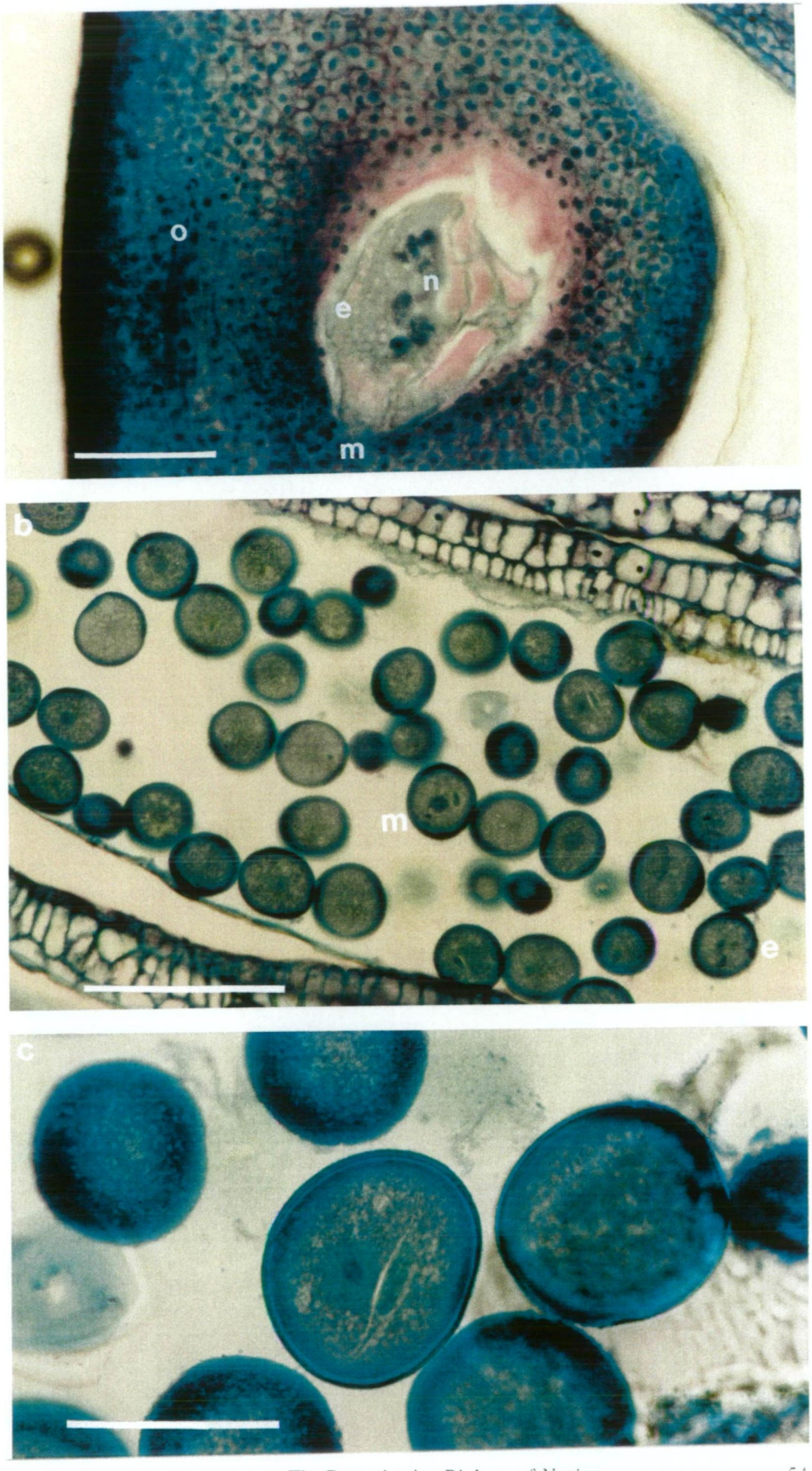
Plate 2.5a Embryo sac formation in *N. filamentosa*. Eight-nucleate embryo sac. (e) = embryo sac, (n) = migrating nuclei, (m) = micropylar end, (o) = ovule. Scale bar = 200μm. (Stain: Toluidine blue.)

Plate 2.5b *In situ N. filamentosa* pollen grains. Pollen grains at both early binucleate (e) and mid-binucleate (m) stage are present. Scale bar = 200μm. (Stain: Toluidine blue.)

Plate 2.5c Mature binucleate pollen grain showing vegetative nucleus and generative cell. Scale bar = 80μm. (Stain: Toluidine blue.)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Species	Generation time	References
Amaryllis belladonna	>6 years	Koopowitz, 1986
Boophane disticha	8 years	Koopowitz, 1986
Brunsvigia litorallis	6 years	Koopowitz, 1986
Clivia hybrids	>8 years	Koopowitz, 1986
Crinum macowanii	>9 years	Koopowitz, 1986
Crinum sp.	5 years	Grove, 1992
Cyrtanthus herrei	8 years	Koopowitz, 1986
Cyrtanthus mackenii	1 year	Koopowitz, 1986
Haemanthus sanguineus	3 years	Koopowitz, 1986
Lycoris spp.	4-5 years	Shii *et al.*, 1997
Narcissus triandrus	3 years	Koopowitz, 1986
Nerine bowdenii	≥4years	Brown, Chapter 2
Nerine masonorum	3 years	Koopowitz, 1986
Nerine platypetala	3-4 years	Craib, 1996
Nerine sarniensis	≥4years	Brown, Chapter 2

Table A.2.1: Generation time of species in the family Amaryllidaceae.

Chapter Three

Horticultural implications of *Nerine* inflorescence development

3.1 Introduction

Preformation of *Nerine* inflorescence buds has several horticultural implications. The lengthy generation time is initially disadvantageous to commercial growers, although once a crop is established a regular yield of flowers should be achievable when regular lifting and replanting is unnecessary. However, the extended period of development results in the inflorescence being susceptible to environmental factors and disturbance over three growing seasons (see Chapter 2), which can jeopardise regular flowering. As consistency in quality and quantity of product is important when establishing a commercial market, irregular flowering, as often seen in *Nerine* (Systema, 1971; Smithers, 1990; Theron and Jacobs, 1992; van Brenk and Benschop, 1993; Vishnevetsky *et al.*, 1997), can be a major problem in a horticultural crop. Therefore, identification of critical stages of inflorescence development and/or environmental factors that cause these events are important to maximise yield of blooms from the bulb crop. It is also important to establish whether disturbance can have an adverse effect on floral quality traits, such as scape length, number of florets and floral morphology.

Early horticulturists (generally unaware of inflorescence preformation) assumed *Nerine* bulbs could be lifted, without harming the developing inflorescence, during a period of dormancy, after senescence of the leaves. However, observations of developing inflorescences of *N. sarniensis* and *N. bowdenii* have shown that inflorescences continue to grow and differentiate during these vegetatively dormant periods (see Chapter 2). It is therefore possible that vegetatively dormant bulbs may be sensitive to disturbance and/or environmental factors, which can trigger floral

abortion, malformation or prevent an inflorescence from being initiated. Whilst disturbance, in the form of bulb lifting or transplanting,, can be used together with storage regimes to enhance flowering, in particular to extend the flowering season, any horticultural regime that requires disturbance may induce irregular flowering or inferior inflorescences in the next or subsequent flowering season. If this is the case, susceptible bulbs should ideally be left *in situ*, being disturbed only when necessary to separate clumps. The climate of Tasmania, Southern Australia, is ideal for such practice, allowing outdoor production of *N. sarniensis* and *N. bowdenii* without the need for lifting and consequent disturbance of the bulbs.

3.2 Materials and methods

3.2.1 Determination of causes of irregular flowering in *Nerine*

Bulbs of *N. bowdenii* x 'Clone 63', *N. sarniensis* x 'Fothergillii major' and *N. sarniensis* x 'Rosea' were selected at random from field grown plants. The number of daughter bulbs were recorded and bulb scales peeled back to determine number of leaves between florets and flowering history.

The study of *N. sarniensis* x 'Rosea' was extended over two years to determine whether the proportion of specific causes of non-flowering were consistent. In both years, 100 randomly selected bulbs of *N. sarniensis* x 'Rosea' were removed from the field in mid-January (summer) and grown in pots placed outdoors with the protection of a wind-break. This cultivar was also used to identify the approximate timing of inflorescence abortion. This was calculated by measuring aborted buds and comparing with measurements of normal buds taken from *in situ* bulbs harvested at monthly intervals (see Chapter 2). For the purpose of this study, it was assumed that inflorescence development had ceased at the time corresponding to a normally developing bud of this size. It is possible that some or all of the aborted buds ceased developing at an earlier stage but the spathe continued to elongate giving the bud a length measurement corresponding with an older bud. However, aborted buds appeared to have reached a stage of floret development that corresponded with normal buds of this size.

3.2.2 Incidence of floral abnormalities in *Nerine*

Bulbs which sent up flowering stems were also examined routinely to observe any evidence of floral malformation. These included stunted scape growth, non-

production of pollen, non-opening of florets and reduction in number of florets per inflorescence.

3.3 Results

3.3.1 Determination of causes of irregular flowering in *Nerine*

Examination of non-flowering bulbs of *N. bowdenii* x 'Clone 63', *N. sarniensis* x 'Fothergillii major' and *N. sarniensis* x 'Rosea' revealed three main causes. These were: (i) bulbs not having reached flowering size; (ii) bulbs that had previously flowered but the inflorescence for the particular year had not been initiated; and (iii) the inflorescence had been formed but had ceased to grow (*i.e.* aborted). Relative percentages of non-flowering bulb attributable to these causes are outline in Table 3.1.

Cultivar	% Bud absent *	% Bud aborted
N. bowdenii x 'Clone 63'	17.5	12
N. sarniensis x 'Fothergillii major'	19	12
N. sarniensis x 'Rosea'	6	9.5

Table 3.1: Percentage of bulbs where inflorescence bud is absent or aborted. * may be due to bulb not having reached size for first flower, or bud not initiated. (Data included in this table was an aggregate of resultsfrom randomly selected bulbs taken from the field over two successive seasons.)

To determine whether the proportion of non-flowering bulbs was consistent from one season to another, a large sample of *N. sarniensis* x 'Rosea' bulbs was observed over two successive seasons (Table 3.2). In 1997, only 77% of *N. sarniensis* x 'Rosea' bulbs produced a flowering stem. This was substantially below the 89% flowering in 1998 and appears to be due to a higher rate of bud abortion in 1997 (Table 3.2).

Year	% Bulb not size	% Bud not initiated	% Bud aborted
1997	4	2	17
1998	2	4	5

Table 3.2: Causes of non-flowering in *N. sarniensis* x 'Rosea' (as a percentage of all bulbs).

Bulbs not of flowering size

In all cultivars, a small proportion (2-4%) of bulbs had not reached the stage of first flowering.

Non-initiation of the inflorescence

Non-initiation of the inflorescence was found in all cultivars examined. In both *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Fothergillii major', the incidence was relatively high (over 10%). In *N. sarniensis* x 'Rosea', a smaller proportion of bulbs exhibited non-initiation, varying from just 2% (1997) to 4% (1998).

Where non-initiation had occurred, there was a corresponding increase in the number of daughter bulbs produced by the mother bulb (Table 3.3; Plate 3.1 a). In *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Fothergillii major', bulbs with no daughter bulbs had a low average number of leaves between inflorescences (Table 3.3), indicating there had been few cases where a bud had not been initiated in a particular growth cycle. This was also the case in bulbs of *N. bowdenii* x 'Clone 63', with 2-4 daughter bulbs, and *N. sarniensis* x 'Fothergillii major' bulbs, with one daughter bulb. However, when greater numbers of daughter bulbs are present, average number of leaves between inflorescence is indicative of non-initiation of inflorescences (Table 3.3). In *N. bowdenii* x 'Clone 63', bulbs with 5-7 daughter bulbs had an average of 12.4 leaves between inflorescences compared to an average of 6.3 leaves when buds are initiated normally (Table 2.1). Similarly in *N. sarniensis* x 'Fothergillii major' with over two daughter bulbs, the average leaf number was 12.5 compared to 6.2 in bulbs of this cultivar when the inflorescence has not been missed. This high average leaf number represents non-initiation in each of these bulbs.

Cultivar	Number of daughter bulbs	Leaves between inflorescences
N. bowdenii x 'Clone 63'	0	7.5±1.5
	1	7.8±0.6
	2-4	7.3±1.3
	5-7	12.4±1.6
N. sarniensis x 'Fothergillii major'	0	5.6±0.4
	1	7.0±0
	2-4	12.5±0.2

Table 3.3: Average number of leaves between inflorescence buds for bulbs with varying number of daughter bulbs.

Floral abortion

Inflorescence abortion can occur at any time, however it mainly occurs during the second season of growth. A significant proportion of aborted inflorescences are 12-15mm in size, which corresponds to their growth having ceased during mid-spring (late September to early November). In *N. sarniensis* x 'Rosea, 79% of aborted buds (1997) and 100 % (1998) were of this size. This points to the period 4-5 months prior to flowering, just prior to the formation of pollen mother cells (PMCs), as a critical point in inflorescence development.

In 1997, there was a small number of buds that had ceased growing at a later stage, approximately one month prior to flowering. At this stage the scape had elongated, but had not emerged from the bulb. Aborted buds at this stage did not occur in any of the *N. sarniensis* x 'Rosea' bulbs in 1998, and occurred only rarely in *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Fothergillii major'.

The loss of inflorescences through abortion was significantly different in the two years of the study. In 1997, the number of floral abortions in *N. sarniensis* x 'Rosea' appeared to be very high, accounting for 74% of the non-flowering bulbs examined. Although this high level did not occur in 1998, floral abortion represented the major cause of non-flowering in this cultivar.

3.3.2 Incidence of floral abnormalities in *Nerine*

Abnormalities in floret initiation and development

The number of florets found in individuals within a single cultivar was highly variable (Table 3.4). Variation in floret number also occurs within a single individual, which is tracked over two successive season (see Chapter 8) suggesting environmental modification of floret number, presumably acting at the time of floret initiation.

Cultivar	Number of Florets
N. bowdenii x 'Clone 63'	4 - 8
N. sarniensis x 'Fothergillii major'	8 - 15
N. sarniensis x 'Rosea'	4 - 9

Table 3.4: Number of florets per inflorescence (range).

Abnormalities in floral organ differentiation

Observed deformities of floral organs include malformed styles, malformed anthers (single whorl only) and fusion of dissimilar organs. These deformities tended to occur only in a single floret, and occurrence was rare.

Abnormalities in gamete formation

The most common floral abnormality was where florets had one whorl of anthers devoid of pollen and the second whorl exhibiting normal pollen production. This was observed more commonly following a period of hot, dry weather, which corresponded to the time of gametogenesis.

Abnormalities during scape elongation

Examination of emerged inflorescences revealed a number of floral deformities that may have been due to a disturbance to the normal development of the inflorescence at the scape elongation stage. These included floral scape abnormalities, where the scape was very short and/or twisted after emergence from the bulb. This was seen in a number of cultivars including *N. sarniensis* x 'Jenny Wren', x 'Rosea' and x 'Rosita'. In many cases, the scape had senesced prior to the opening of the florets. The non-opening of florets (Plate 3.1 b) was another abnormality that apparently occurred late in floral production. In these cases the larger florets opened as normal, but several florets did not elongate after opening of the spathe and senesced prior to opening. In Tasmania, these deformities were more prevalent during the 1997 flowering season which was preceded by unusually hot temperatures in December (1996) and January (1997), at the time when scape elongation was occurring.

3.4 Discussion

The lengthy generation time of *Nerine*, although inconvenient to growers could be withstood if a quality crop with consistent flowering could be established during this time. Unfortunately, some *Nerine* cultivars, in particular those of *N. sarniensis,* commonly exhibit irregular flowering preventing their widespread use as a crop plant (Douglas, 1967; Smithers, 1990; van Brenk and Benschop, 1993). Two primary causes of irregular flowering have been identified, namely non-initiation of the inflorescence and inflorescence abortion. Due to the preformation phenomenon, these events are unable to be identified by growers prior to the time in which flowering would normally occur.

Preformation results in a single inflorescence being rendered susceptible to environmental factors and disturbance over three growing seasons. Interruption in the first season may cause non-initiation of the inflorescence, or may affect the number of florets initiated. In the second season, organogenesis can be affected, whilst in the third season, gametogenesis and scape elongation can be affected. Additionally, floral abortion can occur in any of the three growing seasons. Depending on the nature of the disturbance, it may affect more than one inflorescence within the bulb. Thus, whole crops may have reduced flowering rates for consecutive seasons as a result of a particularly adverse event.

In all cultivars examined in this study, there were bulbs of apparent flowering size that had not initiated a flowering bud for the following year. This was due to either: (i) bulbs not yet achieving sufficient growth for initiation of the first flower; or (ii) bulbs with a flowering history, having missed initiation in a particular year. The bulbs that had not achieved flowering size, had not initiated a bud that particular year and cannot be forced to flower under artificial conditions. It is to be expected a certain percentage of these bulbs will be selected as a result of the normal size variation within the population.

Bulbs having previously flowered, but missing the initiation of an inflorescence, are a result of a breakdown in the normal development cycle and an extreme result of bulb disturbance in the first year of floral development. This condition indicates some disturbance to the bulb at the time a bud would normally have been initiated (two seasons previously). The precise timing of initiation appears to be of vital importance. Once a bud has been 'missed', another will not form until a further full years' growth cycle has been completed. This is evident in bulbs by counting leaf scales in between inflorescences and appears to be a common cause of irregular flowering, accounting for at least one third of non-flowering bulbs that were of flowering size.

An irregular number of leaves (4-15) between inflorescence buds has been reported in *N. flexuosa* 'alba' (Fortanier *et al.*, 1979). The larger numbers found may be attributable to instances where inflorescences have not been initiated due to disturbance or other adverse conditions. Non-initiation could be expected more commonly in *N. flexuosa* 'alba' than *N. bowdenii* and *N. sarniensis* due to its

reportedly shorter period of inflorescence development, and the initiation of more than one inflorescence bud in any one year under suitable growth conditions (Fortanier *et al.*, 1979).

In *N. bowdenii* and *N. sarniensis,* inflorescence initiation occurs in spring (see Chapter 2), so it would be disturbance during this period that could cause non-initiation. In *N. sarniensis*, bulbs are vegetatively dormant at this time, so practices of moving bulbs when dormant may have inadvertently caused non-initiation. When inflorescence initiation has been missed, reproductive effort appears to switch to vegetative propagation as initiation of several daughter bulbs (Plate 3.1) often follows such an event (*i.e.* a meristem present in outer bulb scales is activated). This can occur when bulbs are lifted and/or replanted around late spring, (*i.e.* the time of initiation). The result of such disturbance is a low flowering percentage in two years time. A high incidence of daughter bulb production (with as many as seven per bulb) may alert growers to this situation.

Although inflorescence abortion can occur at any time, with a significant disturbance, there appears to be one stage when the bud is particularly susceptible. In *N. sarniensis* x 'Rosea' the majority of aborted buds, in both years, occurred when a length of 12-15mm had been reached. This corresponds with the size of normal buds during spring of the second year of inflorescence development. At this developmental stage formation of all florets and floral organs has occurred, but the buds have not yet undergone microsporogenesis and megasporogenesis. In both years of this study, a major proportion of the non-flowering bulbs exhibited inflorescence abortion at this stage, which points to 4-5 months prior to flowering as another critical point in the development of the inflorescence. The larger number of spring abortions in 1997, points to a particularly affective disturbance or set of environmental conditions during the 1996 summer.

The physiological cause of floral abortion in *Nerine* is believed to be due to source/sink relationships in the bulbs, which can be affected by stresses, such as lifting (Blake, 1999). Theron and Jacobs (1996) found the inflorescence of *N. bowdenii* was more vulnerable to stress during the first half of the growing season, which corresponds with the results of this study, as spring is the beginning of the growth cycle.

It is noteworthy that this susceptibility of the inflorescence, 4-5 months prior to flowering, corresponds with a period of vegetative dormancy in *N. sarniensis*. These results show that the inflorescence can be disturbed when the bulb appears to be in a state of dormancy. As with non-initiation, this may explain some past incidences of irregular flowering where bulbs have been moved when thought to be dormant, causing subsequent abortion of a developing inflorescence. Disturbance of bulbs, such as by lifting and replanting, or climatic extremes are not the only cause of floral abortions in *Nerine*. Floral abortions can occur following inappropriate storage. Correct storage temperature is critical in decreasing floral abortions (Groen and Kok, 1997).

As florets are initiated sequentially it is quite possible their initiation or development may be prevented by disturbance at a critical time, resulting in a reduction in floret number. This is supported by the large variation in floret number found within a single line. As these lines are clonal, individuals would presumably be of identical genetic makeup, and variation would be due to environmental factors. Florets are initiated during the first year of bud development (see Chapter 2), thus the size of the floral head can be influenced by environment at this early stage.

The floral organs, developing over a two year period in *Nerine* (see Chapter 2), could also be susceptible to environmental influences for this extended period (environmental factors are known to be an underlying cause of some common floral abnormalities such as abnormal stamens [Greyson, 1994]). In *Nerine*, observed deformities included abnormal stamens and pistils. Where these occurred in a single floret only, it is possible that some event has adversely affected the bulb at the time of development of that particular organ and because development of florets and organs within florets is sequential, no others have been affected. By the time floral organs are developing (in the second year of development) significant resources have been allocated to inflorescence development. Consequently, sequential organogenesis is effective in ensuring one disturbance to the bulb does not result in a catastrophic loss of the particular organ type to the whole inflorescence. Although disturbances to floret initiation and organogenesis would have relatively minor effects on the fertility of the inflorescence *in vivo*, these may adversely affect flower quality in a cut-flower crop. Consequently, such disturbances need to be avoided if possible.

The late formation of gametes, in light of the extensive period of inflorescence development, is an interesting feature of *Nerine*. It may be a function of resource allocation, where unnecessary allocation of resources is not committed to a bud that may abort, until it has a reasonable chance of successful flowering. Thus, beginning gamete production during the spathe elongation phase, when the inflorescence has an excellent chance of reaching anthesis, may be of adaptive significance. Progressive and asynchronous gametogenesis may also be an adaptation to ensure that a disturbance during development does not affect an entire inflorescence. A short disturbance during the gamete production phase would affect only some of the individual florets and therefore reproductive function, albeit in a reduced capacity, could continue. Observed cases of inflorescences where some florets have empty anthers and others produce normal pollen could be a result of a disturbance to pollen grain meiosis (PGM) in one floret, with earlier or later PGM in another floret, remaining unaffected.

The pattern of development for *Nerine* inflorescences, in particular the late gamete production, needs to be considered by growers. Importantly, bulbs need to be in adequate growth conditions 3-4 months prior to flowering. Temperature can be critical, with high temperatures during this final phase affecting not only gamete production, but floral form. This may be an important consideration in the event of a hot summer season. In *N. sarniensis*, gamete production occurs during the dormancy phase of the bulb, so lifting and storing of apparently dormant bulbs may adversely affect the fertility of the inflorescence, which is an important consideration for breeders.

Incidence of floral abortion in the spathe elongation phase, when gametogenesis is occurring, is low (although it was found in three *N. sarniensis* x 'Rosea' bulbs in 1997, it was not observed in 1998). Therefore, it is unlikely that an inflorescence at this late stage is particularly susceptible to disturbance. The more likely explanation is that of physical damage to these particular bulbs during the transfer process in 1997. When you consider the input of energy and resources put into an inflorescence that had reached this stage of development (*i.e.* two growing seasons for formation of the florets and constituent reproductive organs, microsporogenesis and the rapid cell division to elongate the scape), it would seem unlikely that this stage would be particularly susceptible to abortion. It is, however, probable that observed floral deformities, such as short scapes and non-opening florets, are due to

a disturbance to the bud at the scape elongation stage. This is supported by the increase in occurrence following particularly hot temperatures in the month before flowering at the time of scape elongation.

Whether late abortion is a result of physical damage to bulbs or disturbance of normal developmental processes, lifting of bulbs at this late stage (mid-summer), does not appear to cause a significant disturbance to the inflorescence. Similarly, Warrington and co-workers (1989) reported a high percentage of bulbs flowering after lifting in late January (and subsequent storage), suggesting late abortion was not a problem in *N. sarniensis* x 'Salmon Supreme'. They also found new inflorescences continued to be initiated in these lifted bulbs. It appears, therefore, that careful disturbance of bulbs may occur at this time without adversely affecting flowering.

3.5 Conclusion

Two critical stages in the development of the *Nerine* inflorescence have been identified: (i) initiation of the inflorescence; and (ii) 4-6 months prior to scape elongation. Both these stages occur in spring.

If bulbs are disturbed (*e.g.* lifted and stored) around the time of inflorescence initiation, a bud may not be initiated, and the bulb will switch to vegetative propagation via daughter bulbs. A new bud will not be initiated until the corresponding time in the following season. Although floral abortion can occur throughout the development process, the inflorescence is particularly susceptible to abortion if bulbs are disturbed during the second season. The majority of incidences of floral abortion occur mid/late spring, 4 - 6 months prior to scape emergence. To overcome the problem of irregular flowering, bulbs need to be left *in situ* where possible to avoid damage to the developing inflorescence, or carefully lifted and replanted in the month before flowering.

Plate 3.1a *N. bowdenii* x 'Clone 63' bulb with multiple daughter bulbs.
Plate 3.1b Inflorescence of *N. sarniensis* x 'Flame' with aborted florets (a). Scale bar = 1cm.

Chapter Four

Stigmatic receptivity, pollen viability and fertilisation in *Nerine*.

4.1 Introduction

Production of a seed, followed by germination of a seedling is the culmination of a number of important and sequential events (Clarke and Knox, 1978; Ladizinsky, 1992; Dodds *et al.*, 1996). These include pollination, pollen germination, pollen tube penetration of the stigma and style, pollen tube entry into the ovary, ovule and embryo sac, and double fertilisation. During these processes, inter-cellular interactions of the mating partners are occurring, which must be accurately coordinated for successful completion (Hogenboom, 1984). Following fertilisation, embryo and endosperm development as well as seed maturation occur. Control of these processes is attributable to parental genomes interacting within the nucleus (Hogenboom, 1973; 1984).

An understanding of the reproductive biology and fertility of the genus of interest is very important to the success of breeding programmes. Breeding system studies, which establish pollen viability, stigmatic and ovular receptivity and optimum pollination time, have the potential to increase effectiveness of controlled breeding programmes (Sedgley and Smith, 1989; Said *et al.*, 1991). Studies of reproductive biology have been completed on many species of commercial interest (*e.g.* *Eucalyptus woodwardii* [Sedgley and Smith, 1989]; *Crocus vernus* [Chichiriccò, 1990]; *Cucurbita pepo* [Nepi and Pacini, 1993]; members of the Proteaceae [Goldingay and Cathew, 1998]; *Dryandra quercifolia* and *D. formosa* [Matthews and Sedgley, 1998]).

There has been scant attention given to the breeding system of *Nerine*, despite hybridisation programmes operating from the mid 19th century. It is appropriate, with programmes for cultivar improvement shifting to a more scientific basis, that attention is given to the determination of factors such as pollen viability and pistil receptivity. Tracking of normal fertilisation may also allow the determination of point(s) of failure in hybrid crosses and assist in devising methods of intervention to overcome crossing barriers (see Chapters 6, 7).

A broader view encompassing the reproductive ecology of the genus, allows consideration of factors such as the longevity of the *Nerine* inflorescence, the sequential opening of the florets and the relative timing of the male and female reproductive phases. The inter-relationship of these factors, together with changes in floral morphology and methods of pollen transfer, influence the relative frequency of self and cross pollination and have implications both for breeders and the conservation of natural populations (*e.g.* O'Brien, 1996). For example, inflorescence longevity, an important characteristic for cut-flowers, is related to the rate of outcrossing and effectiveness of attracting pollinators in natural populations (O'Brien and Calder, 1993).

In the presence of viable (and compatible) pollen, a receptive pistil is necessary for normal fertilisation. The determination of the period of pistil receptivity is an important step towards achieving successful fertilisation in hybridisation programmes (van Went *et al.*, 1984). A limited period of pistil receptivity may cause some crosses to fail or result in low seed set. In the absence of knowledge of stigmatic receptivity, the hybridiser may apply a mixture of pollen to a stigma over the period of floral life. This is not always possible or convenient especially when there are few plants to work with or a large scale breeding program is being undertaken. Consequently, methods that identify stigmatic receptivity are useful for the plant breeder. Three commonly used techniques for assessing receptivity are: (i) presence of esterase in stigmatic tissue (Mattson *et al.*, 1974; Bernhardt *et al.*, 1980); (ii) use of fluorescence microscopy to detect pollen tubes in the style (Sedgley, 1979; O'Brien, 1996); and (iii) morphological changes (Collins and Grey, 1988; Said *et al.*, 1991). Detecting the appearance of callose in pistil tissues has also been suggested as a useful bioassay to determine the culmination of pistil viability (Dumas and Knox, 1983). However, the only reliable method of assessing the above indirect techniques is by direct measurement of seed set.

In this chapter, the pollen tube pathway and normal process of fertilisation, including a timetable of pollen tube growth are described in *Nerine*. The period of optimal pistil receptivity has been determined by sequential pollination of the stigma, and subsequent measurement of seed set. This has been compared with timing of morphological changes in florets after anthesis as well as stigmatic receptivity (as identified by traditional measures; esterase activity and number of pollen tubes present in the style). This has allowed the usefulness of indirect measurements as indicators of receptivity to be assessed in *Nerine*. Finally, the relationship of morphological changes to reproductive ecology is addressed.

4.2 Materials and methods

4.2.1 Floral morphology

Measurements of style length and ovary diameter were carried out prior to pollination. Stigma morphology was examined and photographed under a Zeiss Stemi SV stereo microscope and an Electroscan environmental scanning electron microscope. Styles were embedded and sectioned as described in 2.2.

4.2.2 Determination of self-compatibility

It was essential to track the progress of known compatible crosses when examining the fertilisation process. Self compatible cultivars were identified by bagging field grown plants to exclude non-self pollen, harvesting resultant seeds and observing normal germination.

Comparison of rates of self-pollination and open pollination was achieved by randomly selecting neighbouring plants as controls. These were marked with a twist-tie (as used to secure the bags in bagged plants) and left uncovered. Seeds were treated as per bagged plants.

4.2.3 Pollen germination and pollen tube growth

4.2.3.1 Pollen tube growth *in vivo*

To determine timing of pollen tube growth, *N. sarniensis* x 'Rosea' pistils were pollinated with fresh self pollen, as described above, and harvested after the appropriate time. Excised styles were fixed in EAA (3 parts 70% ethanol: 1 part glacial acetic acid) for four hours before storage in 70% ethanol. Styles were softened by immersion in sodium sulphite solution (10%), rinsed in distilled water,

stored for 1-8 days in decolourised aqueous aniline blue solution (0.1%) in 0.1M tripotassium orthophosphate and mounted in glycerol (adapted from Martin, 1959; Shivanna and Rangaswarmy, 1992; Appendix C.1). Styles were examined using a Zeiss Axiovert 35 inverted microscope fitted with an HBO mercury vapour lamp (excitation filter BP365/12, dichroic beam splitter FT395 and barrier LP 397). Counts were made of: (i) number of pollen grains present on the stigma; (ii) number of germinated pollen grains; and (iii) number of pollen tubes reaching the top, middle and base of the style, ovary area and ovules as well as pollen tubes entering a micropyle.

4.2.3.2 Pollen tube growth *in vitro*

Pollen grains were germinated *in vitro* by inoculating fresh pollen into a liquid medium of Brewbaker's solution (Brewbaker and Kwack, 1963; Appendix C.4) supplemented with 20% sucrose. Cultures were gently agitated and incubated at 25°C. Pollen was removed from the liquid culture at intervals and placed in a well-slide. Pollen was fixed in 10% ethanol, stained with the DNA fluorochrome Hoechst 33258 (Sigma) (0.01% dissolved in distilled water; Appendix C.1) (Shivanna and Rangaswarmy, 1992) and viewed as described in Section 2.2.

4.2.4 Determination of pistil receptivity

4.2.4.1 Measurement of seed set

Hand self pollinations were carried out in known compatible crosses (*N. bowdenii* x 'Clone 63' self pollinated and *N. sarniensis* x 'Rosea' self pollinated) from 6 days before anthesis (A-6)[1] until 12 days after anthesis (A+12). Between 5 and 13 crosses were performed at each stage. The procedure for crossing included emasculation of a previously unpollinated floret, labelling, and covering of the stigma with a small aluminium foil cap until pollination (Plate 4.1a). The foil cap was kept in place until the style senesced to prevent further pollination. Seeds were harvested as they burst through the ovary/fruit wall. Time of pollination, relative to anthesis of the floret, was recorded in all crosses (Appendix B: Breeding Records).

[1] Time is referred to relative to anthesis (A) of the floret (*e.g.* A-3 = 3 days before anthesis; A+3 = 3 days after anthesis).

4.2.4.2 Detection of esterase

Unpollinated stigmata were used for esterase tests. Freshly excised stigmata were placed in a phosphate buffer containing 1% sucrose, 0.25% Fast Blue B with a substrate (α-napthyl acetate [0.05%]) or without substrate for controls (Shivanna and Rangaswarmy, 1992). Scoring was by a subjective assessment of the intensity of staining on the stigma using a scale of 0-5 (0 = no reaction; 1 = light staining confined to the tips of some papillae; 2 = light staining extending down the length of the papillae on part of the stigma; 3 = mid/dark staining extending to base of papillae but not covering an entire lobe; 4 = dark staining extending to base of papillae covering whole lobe; 5 = very dark staining extending the length of the papillae and covering the entire stigma [Plates 4:1 b-c]).

4.2.4.3 Number of pollen tubes in the style

Sequential crosses were performed in *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea', from A-6 until A+12 (see 4.2.4.1). Crosses were repeated three times where possible. The number of pollen grains and pollen tubes reaching each point in the fertilisation pathway was counted using the technique described in 4.2.3.1.

4.2.4.4 Detection of callose

Detection of callose in ovary tissue was by use of aniline blue fluorescence as described in 4.2.4.1.

4.2.5 Pollen longevity

Pollen viability was determined by *in vitro* culture as described in 4.2.3.2. Samples of pollen culture were removed after 48 hours using a pasteur pipette, fixed using 10% ethanol and placed on a microscope slide for observation (Shivanna and Rangaswarmy, 1992). Pollen of different ages, from both *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea', was germinated *in vitro* to determine pollen longevity (five separate plants were sampled each day).

4.2.6 Open pollination

All bulbs in the collection were examined for incidence of open pollination. All florets that were not subjected to controlled crosses were regularly checked for evidence of open pollinations. Any seeds were harvested when they burst through the ovary/fruit wall, measured and placed in seed-raising mix. At floral senescence, all florets were rechecked for possible open pollination, and any ovules that appeared green and/or swollen were removed and placed in tissue culture (see Chapter 7).

Field grown *N. bowdenii*, *N. sarniensis* x 'Rosea' and *N. bowdenii* x 'Clone 63' as well as plants of *N. masonorum*, *N. sarniensis* x 'Jill' and *N. sarniensis* x 'Rosea' grown under plastic tunnels were harvested to assess open pollination rate. Number of seeds per floret and inflorescence were counted.

To determine whether the relatively low seed set on open pollinated inflorescences was due to physical or biological constraints, all florets in the inflorescence of 12 *N. sarniensis* x 'Rosea' bulbs were hand pollinated at A, A+4 and A+8. The number of seeds produced by each floret, relative to its position in the opening sequence, was also recorded.

4.3 Results

4.3.1 Floral morphology

Anthesis of the inflorescence occurs after a period of rapid scape elongation (see Chapter 2), with the spathe valves opening to reveal florets of varying bud length, which will open sequentially. The longevity of the inflorescence, from the opening of the first floret to the senescence of the last floret, can be over three weeks with individual florets lasting up to two weeks before showing signs of senescence.

The *Nerine* style is hollow, with a central channel lined with transmitting tissue (Plate 4.1d). This channel appears early in gynoecial development, prior to the formation of the ovules (Plate 2.2c). The inferior ovary is three-locular having central placentation, with the transmitting tissue being continuous into the placental tissue (Plate 4.1e). The stigma is dry and covered with unicellular finger-like papillae which are tightly packed at anthesis. The stigma changes from its initial cylindrical form to become markedly trilobate with the papillae erect and spreading at A+7 to A+10 (Plates 4.2a-c). This spreading of the papillae greatly increases the surface area of the stigma and exposes the transmitting channel (Plate 4.2d). The number of pollen grains retained on the stigma increases with the opening of the stigmatic lobes.

The length of the style at anthesis varies between *Nerine* cultivars (Plates 4.3a-b). It can be in one of three positions: (i) short, below the level of the anthers (*e.g. N. bowdenii*, *N. flexuosa* 'alba', *N. masonorum*); (ii) exserted from the bud (*e.g. N. sarniensis* x 'Rosea', *N. sarniensis* x 'Fothergillii major'); or (iii) level with the

anthers (*e.g. N. sarniensis* x 'Jill'). In all cases, elongation of the style occurs after anthesis. In short-styled cultivars the elongation period is lengthy, taking almost two weeks to reach maximum stylar length (Figs 4.1-4.3; Plate 4.3c). At this stage, the style is significantly longer than the anthers and protrudes from the floret. In exsert- and level-styled cultivars elongation ceases at A+3 to A+5 (Figs 4.4-4.5; Plate 4.3d).

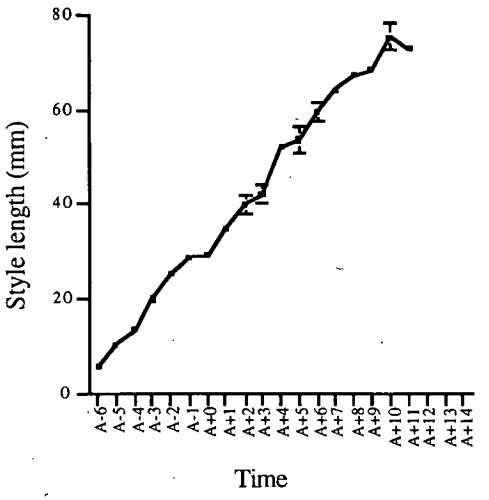


Figure 4.1: Style length increase over time in *N. bowdenii* (a short-styled species).

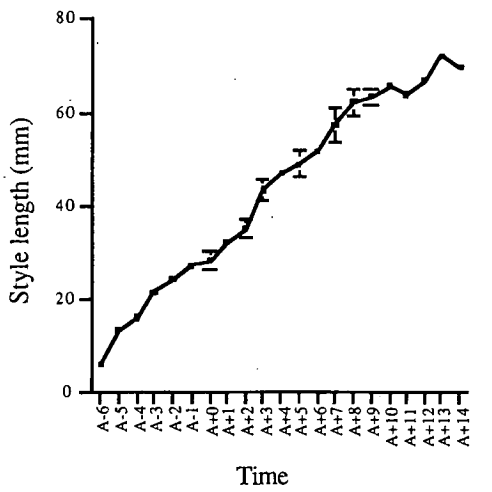


Figure 4.2: Style length increase over time in *N. bowdenii* x 'Clone 63'(a short-styled cultivar).

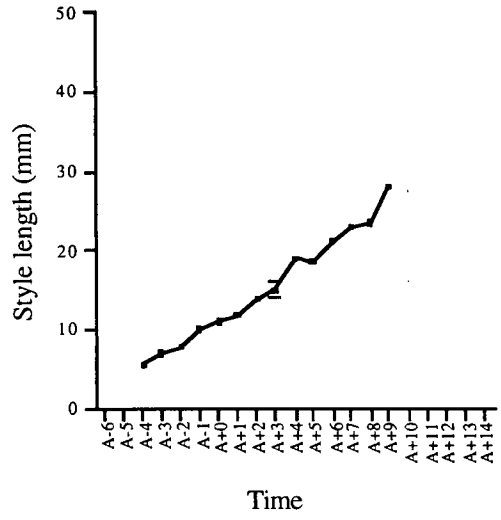


Figure 4.3: Style length increase over time in *N. flexuosa* 'alba' (a short-styled cultivar).

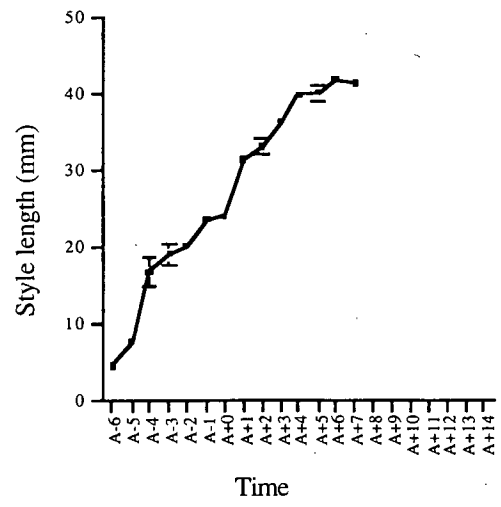


Figure 4.4: Style length increase over time in *N. sarniensis* x 'Jill' (a level-styled cultivar).

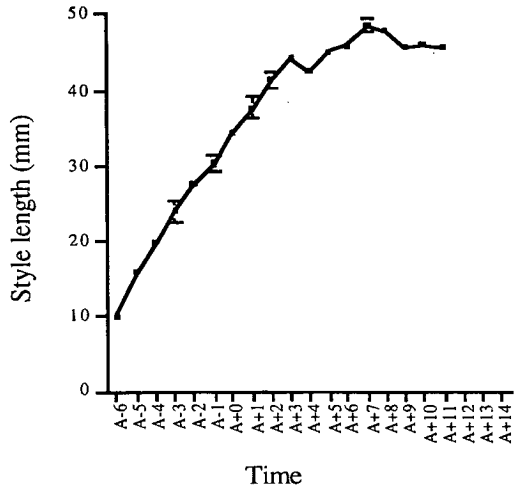


Figure 4.5: Style length increase over time in *N. sarniensis* x 'Rosea' (an exsert-styled cultivar).

Increases in style length are accompanied by other morphological changes. Initially growth of the tepals continues, ceasing at approximately A+5 (Figs 2.3; 2.6). Similarly, ovary diameter increases with maturation of the floret, reaching a maximum at A+4 in *N. bowdenii* x 'Clone 63' and A+3 in *N. sarniensis* x 'Rosea'. In *N. bowdenii*, concurrent with style elongation is an alteration in the colour of the style with age; it progressively becomes darker from base to tip. At anthesis, the pistil of level- and exsert-styled cultivars are in close proximity to the anthers, however there is spatial separation of anthers and pistils in short-styled cultivars. In the latter, elongation of the style brings the stigma close to the anthers from A+5 to A+7.

Anthers are positioned in two whorls, with dehiscence of the outer-most whorl occurring first. The process of dehiscence begins by a splitting of the anther down the central suture of both lobes. The fissure begins at the top and the anther wall progressively curls backwards and inwards to expose the pollen grains (Plate 4.4a). The duration of the opening varies from minutes to several hours and is accelerated by warmer temperatures. Dehiscence is sequential and timing varies between cultivars. It occurs prior to anthesis in some cultivars including *N. bowdenii* x 'Winter Cheer', *Nerine* x 'Fletcherii', *N. sarniensis* x 'Cynthia Chance', and x 'Jill', however, this is not usual. In *N. sarniensis* x 'Rosea', the first anther opens between A and A+2 while the remainder open sequentially from A+2 to A+5. In *N.*

bowdenii x 'Clone 63', the first and last anthers do not dehisce until between A+ 2 and A+4, and up to A+9, respectively.

4.3.2 Determination of self-compatibility

The varieties *N. bowdenii* x 'Clone 63', *N. flexuosa* 'alba' and *N. sarniensis* x 'Rosea' were found to be self-fertile to sone degree, with seeds being produced when individual plants were bagged to exclude non-self pollen. It should be noted, however, that in all cases rate of seed set following open pollination was higher than in bagged plants. The rate of self pollination in bagged plants was much greater in the short-styled *N. bowdenii* x 'Clone 63' (50%) than in the level-styled *N. flexuosa* 'alba' (16%) and the exsert-styled *N. sarniensis* x 'Rosea' (6%) (Table 4.1).

Cultivar	% Florets with seed	
	SELF	OPEN
N. sarniensis x 'Rosea'	6 (78)	9 (120)
N. flexuosa 'alba'	16 (62)	81 (124)
N. bowdenii x 'Clone 63'	50 (46)	54 (39)

Table 4.1: Self pollination rate. Percentage of florets bearing seed from self pollinations in bagged plants where non-self pollen has been excluded (total number of florets in parentheses). Control plants were open pollinated.

4.3.3 Pollen germination and pollen tube growth

4.3.3.1 Pollen tube growth *in vivo*

A time line of fertilisation in *Nerine*, relative to pollen tube position, is shown in Figure 4.6. After pollination, fertilisation takes approximately three days. Pollen grain germination on the stigma can take 2-12 hours. Pollen tubes grow between papillae (Plate 4.4 b-c) and enter the top of the style within 24 hours. Pollen grain germination is not restricted to the central area of the stigma - it can also occur along the stigmatic lobes. Once inside the stigma, most pollen tubes appear to grow towards the centre and progress down the central transmitting channel (Plate 4.4d). The base of the style is reached after approximately 40 hours (Table 4.2) and in the subsequent 12 hours, pollen tubes have entered the ovary and spread along the placentae to the ovules. The culmination of these events occurs after 60 hours when pollen tubes are observed in the micropyles.

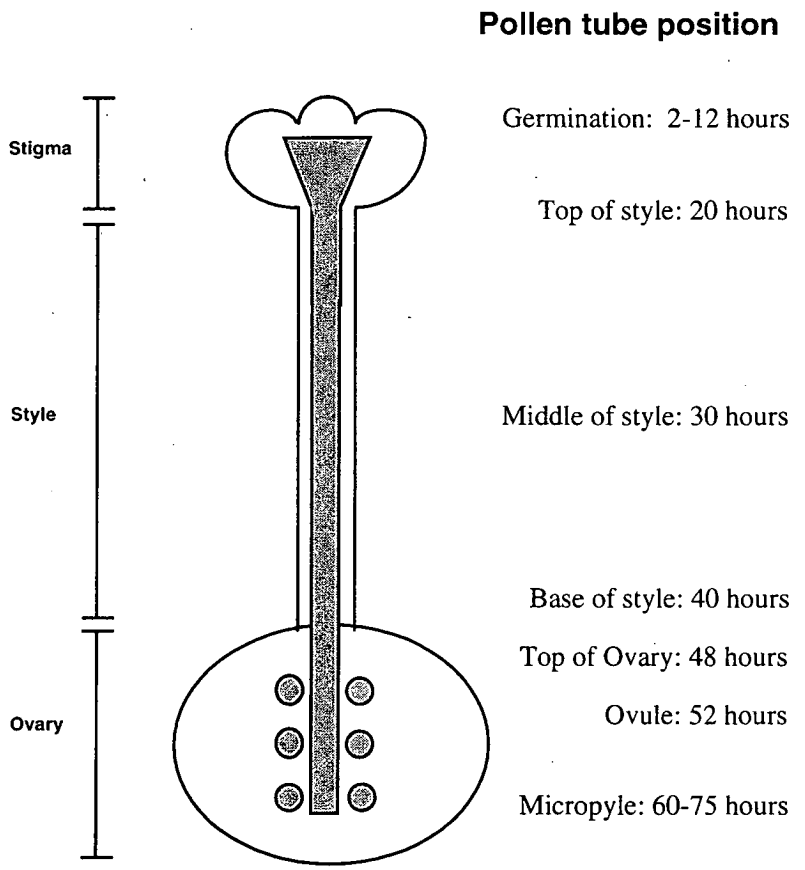


Figure 4.6: Timing of pollen tube progress in *N. sarniensis* x 'Rosea' from pollination (0 hours) to entry of the pollen tube into the micropyle.

Time	Germinated Pollen	Style			Ovary	Ovule	Micropyle
		Top	Mid	Base			
> 8 hours	33	0	0	0	0	0	0
1 day	100	83	50	16	16	0	0
2 days	100	100	100	100	100	58	29
3 days	100	100	100	100	100	75	50
4 days	100	100	100	100	100	100	100

Table 4.2: Percentage of *N. sarniensis* x 'Rosea' styles with pollen tubes at various developmental/positional stages.

Although many pollen grains germinate at the stigma surface, only some reach the bottom of the style (Plate 4.4e). It is the stigma/style interface which provides the greatest barrier to the progress of the pollen tubes, with many pollen tubes either not penetrating or arresting at the top of the style. The majority of pollen tubes reaching the mid-style region continue to grow to the base of the style. Again, the majority of pollen tubes reaching the style/ovary interface enter the ovary tissue, often leading to a greater number of pollen tubes than ovules.

4.3.3.2 Pollen tube growth *in vitro*

Mature pollen grains are binucleate (Plate 4.5a). When pollen tubes grow to 2-3 times the diameter of the pollen grain, the vegetative nucleus enters the pollen tube. Following the entry of the vegetative nucleus, the generative cell enters the pollen tube, undergoing division at the proximal end of the tube (Plate 4.5b). The DNA of the vegetative nucleus is dispersed and travels down the tube in a loose complex while the two sperm nuclei have densely compacted DNA (Plate 4.5c). The male germ unit is situated well behind the cytoplasmic tip (Plate 4.5d) in the early stages of growth. As pollen tubes reach their maximal length the male germ unit moves closer to the distal end with the vegetative nucleus initially leading the sperm cells down the pollen tube. At a later stage, the two sperm cells are positioned at the distal end of the pollen tube. This second position is apparently facilitated by the flattening of the vegetative nucleus thereby allowing the two sperm cells to pass (Plate 4.5e).

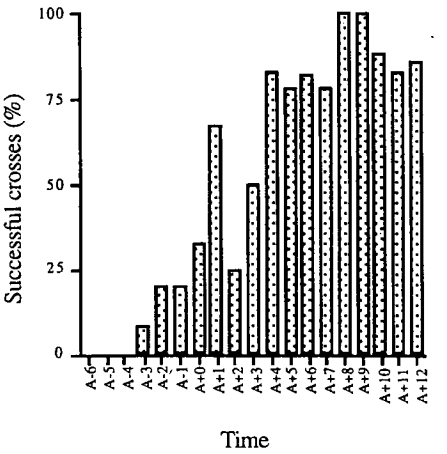
4.3.4 Determination of pistil receptivity

An important aim of this project was to compare direct and indirect assessment of pistil receptivity in *Nerine*. Receptivity was assessed by the indirect measurements of: (i) esterase activity; (ii) number of pollen tubes in the style; and (iii) callose appearance. Additionally, pistil receptivity was measured directly by actual seed set to determine the accuracy and usefulness of the above-mentioned indirect methods in *Nerine*.

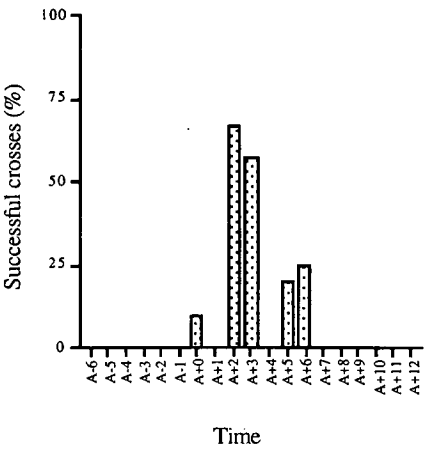
4.3.4.1 Measurement of seed set

Seed was set from pollinations occurring between A-5 and A+12 (Table A.4.1). Of 28 known fertile cultivars, all except one (the triploid *N. sarniensis* x 'Fothergillii major') set seed from pollinations occurring between A and A+5 (Table A.4.1). The number of crosses producing seed in *N. bowdenii* x 'Clone 63', *N. sarniensis* x 'Jill' and *N. sarniensis* x 'Rosea' is presented in Figure 4.7. High numbers of seeds produced per cross coincided with the period in which most crosses were successful; A+4 to A+5 in *N. sarniensis* x 'Rosea', A+8 to A+9 in *N. bowdenii* x 'Clone 63' and A+2 to A+3 in *N. sarniensis* x 'Jill' (Fig. 4.8).

a.



b.



c.

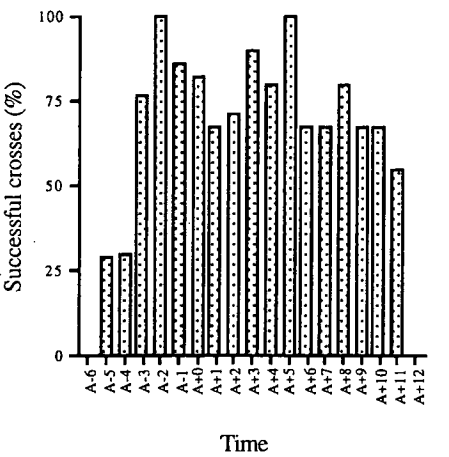
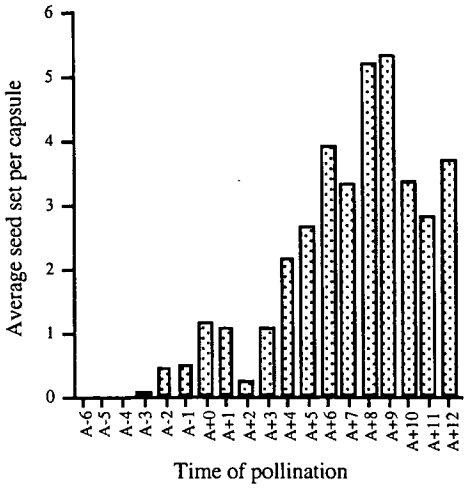
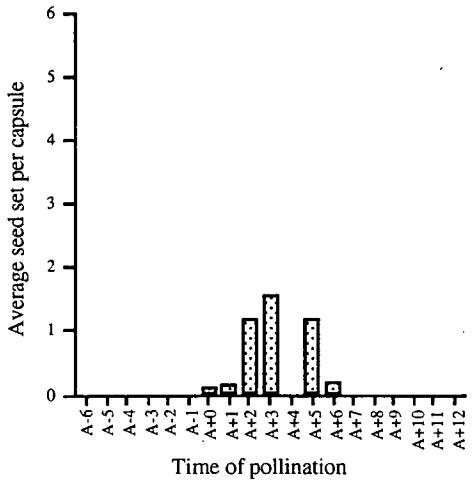


Figure 4.7: Percentage of successful crosses performed at daily intervals A-6 to A+12 days in: a) *N. bowdenii* x 'Clone 63'; b) *N. sarniensis* x 'Jill'; and c) *N. sarniensis* x 'Rosea'.

a.



b.



c.

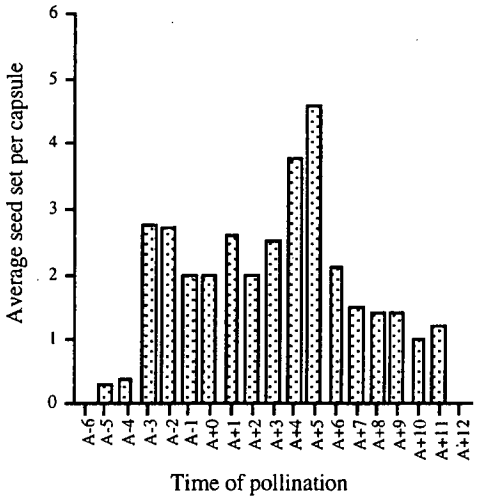


Figure 4.8: Average seed set per capsule in crosses performed at daily intervals from A-6 until A+12 in: a)*N. bowdenii* x 'Clone 63'; b) *N. sarniensis* x 'Jill'; and c) *N. sarniensis* x 'Rosea'.

The pattern of seed set was dramatically different between cultivars. In *N. bowdenii* x 'Clone 63', the period of high receptivity (over 60% of crosses setting seed) was from A+4 to A+12, with peak receptivity occurring from A+8 to A+9 (Fig. 4.7a). At these peaks, which is relatively late in the life of the floret, all crosses were successful, with up to 10 seeds per capsule produced. This contrasts to the period prior to anthesis when the gynoecium was of very limited receptivity. No crosses were successful before A-3, and from this time until anthesis the success rate was very low. Seed set in over 60% of crosses occurred in crosses performed at A+1, but consistent seed set at or above this level did not occur until after A+4.

The period of high seed set in *N. sarniensis* x 'Rosea' was considerably longer than in *N. bowdenii* x 'Clone 63', lasting a period of 14 days and beginning prior to anthesis (Fig. 4.7c). *N. sarniensis* x 'Rosea' set seed from crosses performed as early as A-5, with a high success rate (> 60%) from A-3 until A+10. Two peaks, where all crosses were successful, occurred at A-2 and A+5. Best results in mean number of seeds produced per cross occurred at A+4 and A+5. *N. sarniensis* x 'Jill' was similar to *N. bowdenii* x 'Clone 63', in that receptivity did not occur until after anthesis (Fig. 4.7b). The peak period of receptivity in this cultivar was at A+2 and A+3. It was only at this time that the rate of successful crosses rose above 50%.

4.3.4.2 Detection of esterase

In contrast to actual seed set, esterase activity patterns are very similar in *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea' (Fig. 4.9). Esterase activity level is low before and in the two days after anthesis, and increases to reach a maximum level after A+8 (*N. bowdenii* x 'Clone 63') and A+5 (*N. sarniensis* x 'Rosea'). This high level of esterase activity is consistent in older stigmata and remains until stigmata begin to senesce. Senescence is marked by a browning of the stigmatic papillae, and usually occurs lobe by lobe. Papillae on lobes yet to turn brown record a high esterase reading (approximately 4), but no esterase reaction is detectable on senescing lobes. In both cultivars, esterase activity at each stage shows individual variation and this is greater before, and immediately after, anthesis.

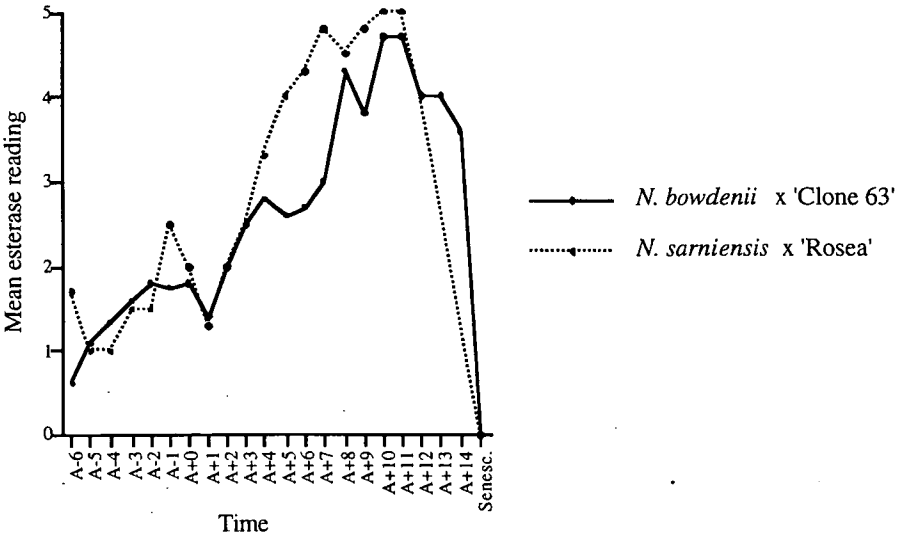
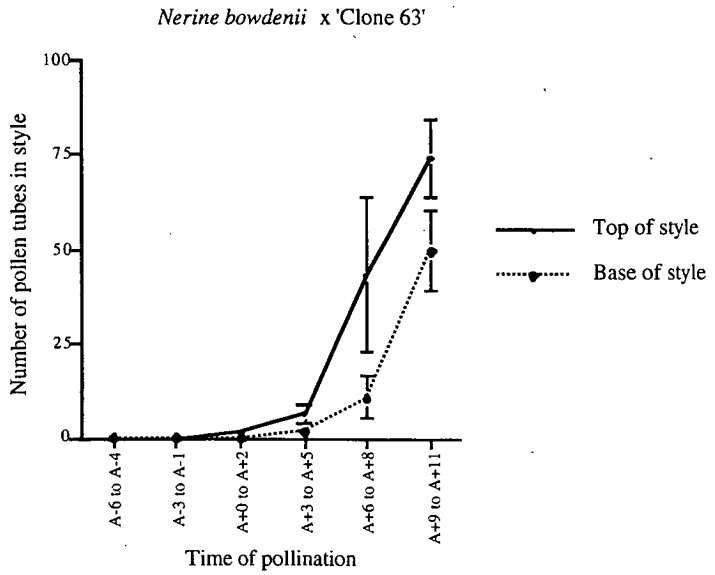


Figure 4.9: Mean esterase reading over time (0 = no reaction, 5 = maximum reaction; see 4.2). Senesc. = senescence.

4.3.4.3 Number of pollen tubes in the style

The presence of pollen tubes in the style also followed a similar pattern in both cultivars, increasing gradually after anthesis and reaching a maximum at A+9 to A+12 (Fig. 4.10). The curve for number of pollen tubes found in the base of the style mirrored that of pollen tube number at the top of the style but with proportionally less pollen tubes reaching the base. There was no evidence of burst or branched pollen tubes or abnormal callose deposits in pollen tubes, which would be indicative of incompatibility reactions. In both cultivars, few pollen grains adhered to pre-anthesis stigmata. Pollen tubes were observed earlier in the style of *N. sarniensis* x'Rosea' styles (A-5), when compared to *N. bowdenii* x 'Clone 63' styles (A-2). In the former, pollen grains germinated on the stigma as early as A-6, yet the pollen tubes did not penetrate the papillae to enter the style. In *N. bowdenii* x 'Clone 63', pollen tubes were not found in the base of the style until A+6, whereas they were detected in the base of *N. sarniensis* x 'Rosea' as early as A-2.

a.



b.

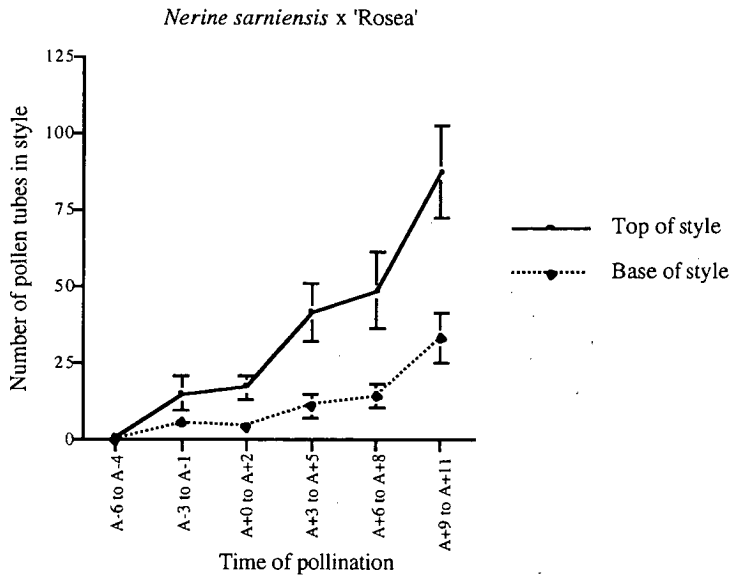


Figure 4.10: Number of pollen tubes found at the top and base of the style in self pollinations of a) *N. bowdenii* x 'Clone 63' and b) *N. sarniensis* x 'Rosea'.

4.3.4.4 Detection of callose

The presence of callose in *Nerine* ovules coincided with the period of maximal pollen tube penetration. However, it was not determined whether callose originated

from pistil tissues or was produced by arrested pollen tubes that were unable to effect fertilisation. Further investigation with known unpollinated pistils is required.

4.3.5 Pollen longevity

The pollen of *N. sarniensis* x 'Rosea' had a high *in vitro* germination rate for both 1-and 2-day old pollen, 93.6% and 72.6% respectively (Table 4.3). Pollen viability dropped sharply after this time, with germination of 3-day old pollen measured at 9.4%. In *N. bowdenii* x 'Clone 63', the *in vitro* germination rate of 1-day old pollen was 62.5% and 2-day old pollen was 94%. Again, germination rate dropped sharply after two days, with 3-day old pollen at 8.8% (Table 4.3).

Days after dehiscence	Mean % viability *N. sarniensis* x 'Rosea'	Mean % viability *N. bowdenii* x 'Clone 63'
1	93.6±1.3[a]	65.2±15.3[a]
2	72.6±10.3[a]	94.0±0.9[a]
3	9.4±4.2[b]	8.8±5.3[b]
4	14.4±6.7[b]	1.2±0.5[b]

Table 4.3: Mean percentage pollen viability up to four days after anther dehiscence. Means with different superscripts are significantly different at the 95% level.

4.3.6 Open pollination

Results of open pollination data collected in 1995-1998 are summarised in Table A.4.2 and Figure 4.11. Open pollination rates for individual years are contained in Table A.4.3. Cultivars with a number of bulbs flowering (*i.e. N. bowdenii*, *N. filamentosa*, *N. flexuosa*, *N. sarniensis* x 'Fothergillii major', *N. sarniensis* x 'Rosea', *N. sarniensis* x 'Jill', *Nerine* x 'Salmonea' and *N. undulata* x 'Roseo-crispa') were analysed in more detail, which permitted between year comparisons. Of these cultivars, *N. filamentosa* and *Nerine* x 'Salmonea' did not set seed from open pollinations in any year. Several cultivars that flowered in only one season also did not set seed from open pollination (Table A.4.3). When this information is considered in conjunction with fertility data from controlled crossing (see Chapter 6), it appears to indicate infertility (see Chapter 6).

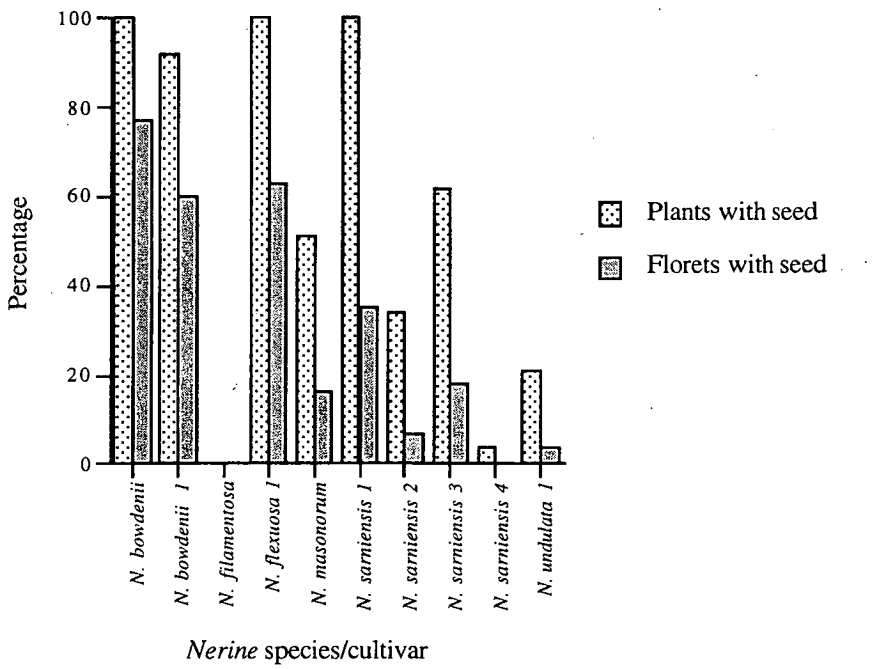


Figure 4.11: Rate of seed set in 6 cultivars of *Nerine*.
Key: *N. bowdenii* 1 = *N. bowdenii* x 'Clone 63';
N. flexuosa 1 = *N. flexuosa* 'alba';
N. sarniensis 1 = *N. sarniensis* x 'Corusca';
N. sarniensis 2 = *N. sarniensis* x 'Jill';
N. sarniensis 3 = *N. sarniensis* x 'Rosea';
N. sarniensis 4 = *N. sarniensis* x 'Fothergillii major';
N. undulata 1 = *N. undulata* x 'Roseo crispa'.

The pattern observed for open pollination was similar to that seen in self pollination trials (Tables 4.1, 4.4). The short-styled varieties, *N. bowdenii* and *N. bowdenii* x 'Clone 63', had extremely high rates of open pollination, 100% and 95% respectively. This high rate is also reflected in percentage of individual florets bearing seed (*N. bowdenii* [77%] and *N. bowdenii* x 'Clone 63' [60%]) (Table 4.4). *N. flexuosa* 'alba', another short-styled cultivar, also had an extremely high rate of seed set, both on plants and individual florets (Fig. 4.11). It should be noted, however, that *N. flexuosa* 'alba' commonly forms parthenogenetic seeds (see Chapter 5).

Stigma Type	Cultivar	% Plants with seed	% Florets with seed
Exsert	*N. sarniensis* x 'Rosea'	62 (60)	15 (324)
Level	*N. sarniensis* x 'Jill'	33 (93)	7 (813)
Short	*N. bowdenii*	100 (37)	77 (213)
Short	*N. bowdenii* x 'Clone 63'	95 (41)	60 (190)
Short	*N. masonorum*	53 (73)	16 (528)

Table 4.4: Open pollination rate. Percentage of plants and individual florets bearing seed from open pollinations (total numbers in parentheses).

The short-styled *N. masonorum* had a lower open pollination rate, with 53% of plants bearing seed (Table 4.4). The flowering period of this species lasted for ten weeks, with the proportion of plants bearing seed averaging 33% for the first nine weeks and escalating to over 60% in the final week. The numbers of seeds per plant showed a significant increase in weeks 5-7, which represents pollination having occurred at the time when the majority of the population was either flowering, in bud or beginning floral senescence (*i.e.* the floral display was at its peak) (Fig. 4.12).

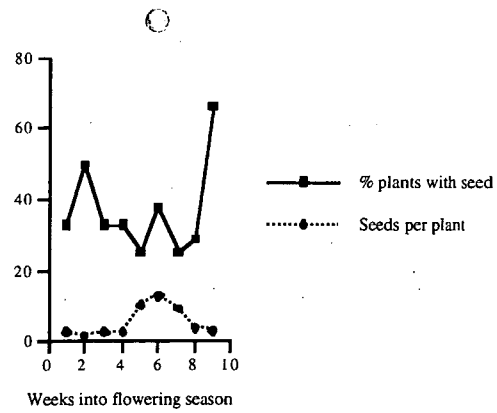


Figure 4.12: Seed set in *N. masonorum* with progress through the flowering season.

N. sarniensis, the cultivars of which have exsert or level styles, generally had a lower percentage of plants bearing seed than the *N. bowdenii* cultivars. *N. sarniensis* cultivars x 'Rosea' and x 'Jill' set seed on 62% and 34% of plants respectively, and had an even lower success rate of seed set on individual florets. *N. sarniensis* x 'Corusca', although setting seed on 100% of plants, had a low rate of seeds per individual floret (Table A.4.1). This phenomenon is also seen in other *N. sarniensis* cultivars (Fig. 4.13).

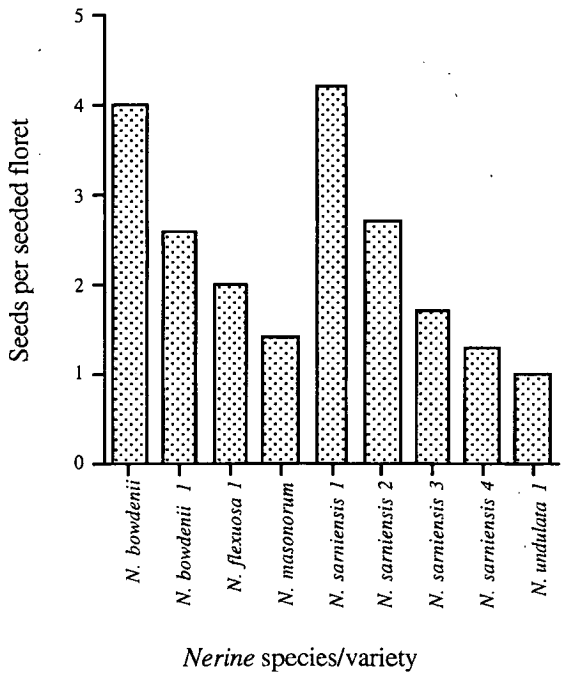


Figure 4.13: Number of seeds set per seeded floret in 5 species of *Nerine*.
Key: *N. bowdenii* 1 = *N. bowdenii* x 'Clone 63';
N. flexuosa 1 = *N. flexuosa* 'alba';
N. sarniensis 1 = *N. sarniensis* x 'Cynthia Chance';
N. sarniensis 2 = *N. sarniensis* x 'Gold Dust';
N. sarniensis 3 = *N. sarniensis* x 'Jill';
N. sarniensis 4 = *N. sarniensis* x 'Eve';
N. undulata 1 = *N. undulata* x 'Roseo crispa'.

To assess whether the rate of open pollination differed in field or tunnel grown plants, inflorescences of *N. sarniensis* x 'Rosea' from both sources were examined for open pollinations in 1997. There was a substantial difference in percentage of plants with seed; 59% of field grown bulbs bearing open pollinated seed in comparison with just 31% of tunnel grown plants (Table 4.5).

Cultivation conditions	% Plants with seed
Field	59 (17)
Tunnel	31 (26)

Table 4.5: Percentage of *N. sarniensis* x 'Rosea' bulbs with seed from open pollinations. Total number in parentheses.

Inflorescences of *N. sarniensis* x 'Rosea', in which each floret was hand pollinated, set seed at a much higher rate (100%) than open pollinated plants (60%). The success rate for individual florets was also greater (84% and 15% respectively), with over half the hand pollinated plants producing seeds in all florets. The number of seeds produced per inflorescence was also greater in the hand pollinated plants, varying between 10-19 compared to a range of 0-3 seeds per open pollinated plant.

In the presence of unlimited pollen, florets in a single inflorescence have differing yields of seeds produced per capsule depending upon position of the floret in the opening sequence. Analysis of seed set in controlled hand pollinations of *N. sarniensis* x 'Rosea' with seven florets, showed highest mean yield was in the fourth floret (3.2±0.5), followed by the third (2.6±0.45), with the first two and last three florets having mean yields of less than two seeds per capsule (Fig. 4.14).

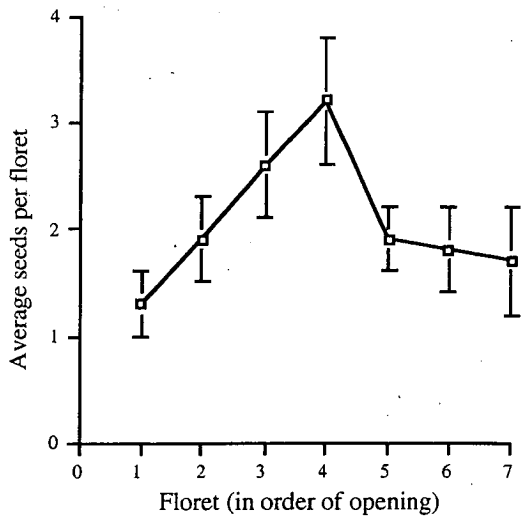


Figure 4.14: Hand pollinated *N. sarniensis* x 'Rosea'

Open pollinated inflorescences of *N. bowdenii* x 'Clone 63' also showed differences in yield depending on position of floret in the opening sequence. The first floret to open produced no seeds. Yield increased up to and including the fourth floret, with florets 3-5 producing approximately two seeds per floret (Fig. 4.15). Hand pollinated *N. bowdenii* x 'Clone 63' inflorescences with five florets showed highest mean seed yield in florets 3-4, with the second and fifth florets yielding an average of one seed. As in open pollination, no seed was set in the first floret to open (Fig. 4.16).

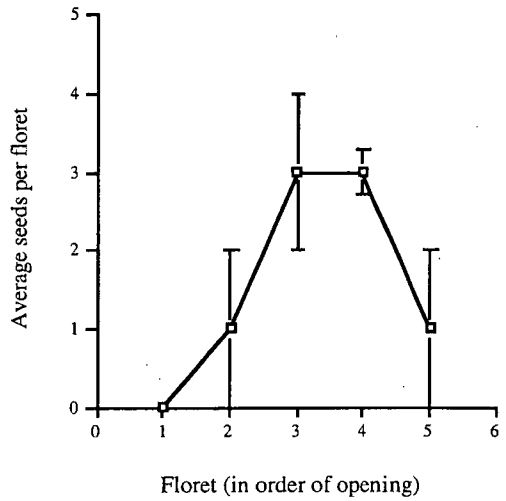


Figure 4.15 : Hand pollinated *N. bowdenii* x 'Clone 63'.

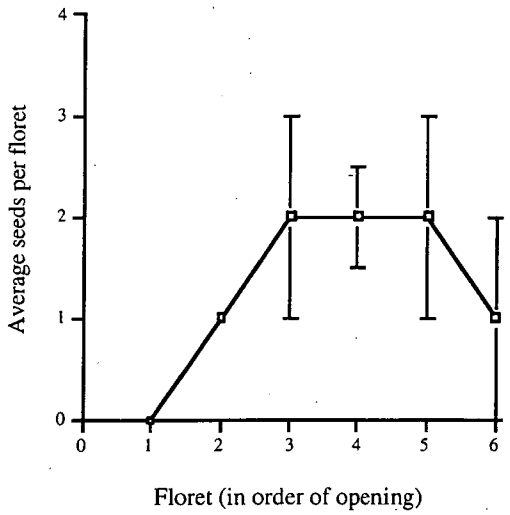


Figure 4.16: Open pollinated *N. bowdenii* x 'Clone 63'.

4.4 Discussion

In *Nerine*, fertilisation occurs within three days of pollination. After being transferred to the stigma, pollen grains germinate within eight hours, grow down the central transmitting channel and enter the micropyle after approximately 60 hours. Pollen tube numbers are greatly reduced at the stigma/style interface, an area in which the pollen tubes need to grow between papillae before making their way into the central transmitting channel. This stylar morphology is common in the monocotyledons and hollow styles, at least in the lower half, are also found in *Cyrtanthus, Crinum, Pancratium* and *Hippeastrum* (Johri, 1966).

Pollen tubes that survive to grow down the central transmitting channel include those which germinated on the edges of the stigmatic lobes and in close proximity to the channel. It appears that some form of pollen tube competition must occur at the stigma/style interface and survival is not solely due to position of deposition on the stigma. Reduction in numbers of pollen tubes with progress down the style appears to be due to normal pollen tube competition, with this phenomenon found in other species. As suggested for other plant species this may be controlled by the female parent (Sayers and Murphy, 1966; Tomer and Gottrich, 1975; Sedgley, 1979).

The number of pollen tubes entering the ovary can be many more than the number of ovules available for fertilisation. In most cultivars of *Nerine*, the number of seeds reaching maturity in each capsule is low (1-3), although there may be over 30 ovules per capsule depending on species. Certainly not all ovules develop into viable seed and the production of high numbers of seeds is uncommon. Therefore it appears pollen availability is not a limiting factor, so number of seeds produced per capsule may be limited by resources of each floret. This is supported by the results of hand pollinations where, even in the presence of unlimited pollen, not all ovules develop into seeds. Although a number of ovules may be fertilised, only some are able to complete development into viable seed. This may be due to a limitation of maternal resources causing very early seed abortion. A similar scenario of early abortion has been demonstrated in *Pisum* (Selkirk *et al.*, 1986).

The timing of pollination influences the number of seeds set per capsule. In both *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea', numbers of seeds set per capsule coincided with peak period of receptivity (when the majority of crosses were successful). This suggests physiological maturity of the ovules may be important.

However crosses occurring later, when physiological maturity has been reached, do not produce such high numbers of seeds despite high numbers of pollen tubes found in the ovary. This may be due to a decrease in available maternal resources for developing seeds that have been fertilised later in the life of the floret and supports the notion that seed abortion will occur when resources are limited.

Consideration of pollen viability and longevity is vital to the success of controlled crosses. If pollen fails to mature or loses viability prior to stigmatic receptivity, it is unable to effect fertilisation. Pollen viability data indicates freshly dehisced pollen, less than two days old, should be used for optimal success. It should be noted, however, that viability results were for *in vitro* conditions and *in vivo* germination could be greater than the results indicate. The lower percentage germination of three day old or older pollen may have been due, at least in part, to a population effect. As fewer grains are present on the anther, the density of the pollen germination medium is much less. Smaller populations can show unsatisfactory germination due to insufficient concentration of pollen growth factors (Shivanna and Rangaswarmy, 1992). For confirmation of this result, the ability of pollen of varying ages to effect seed set would need to be tested. This is the most accurate test of viability (Smith *et al.*, 1984; cited in Ramsey and Vaughton, 1991). However, if viability is correlated with time on the anthers, the results obtained are supported, as most pollen has been dispersed prior to day three. In general, pollen exposure is correlated with viability (Nepi and Pacini, 1993), as keeping anthers intact for longer than the period of viability is wasteful of resources. This has been confirmed in *Leptospermum* where pollen viability remains high for the duration of the period in which it is presented to pollinators (O'Brien and Calder, 1993).

The observed interaction between the sperm cells and the tube nucleus in *in vitro* pollen germination raises some interesting questions. Whether the sperm cells actually overtake the vegetative nucleus in the lower half of the pollen tube *in vivo*, and whether this occurs in all pollen tubes prior to fertilisation, remains to be determined. If the observations reflect *in vivo* phenomena, the time taken for pollen tube growth prior to arrival at the micropyle and/or the distance travelled may be critical to allow this overtaking process to occur. This may have practical applications when interventionist techniques, such as style length reduction, are employed in crosses (see Chapter 7).

In order to achieve a successful seed producing cross, determination of the period of pistil receptivity can be critical (see 4.1). Fertilisation can occur prior to anthesis in *N. sarniensis* and *N. bowdenii* cultivars, and extends to A+12 in some cases. However, in all, except one, of the cultivars used in controlled crossing experiments, seed has been set between A and A+5. The exceptional case was *N. sarniensis* x 'Fothergillii major', a triploid in which seed set was infrequent due to reduced fertility. Consequently, it may be unrepresentative of diploid *Nerine* cultivars.

In the three cultivars of *Nerine* where detailed seed set data were collected, very different patterns in the onset and duration of pistil receptivity are demonstrated. The time of peak receptivity varied from A+2 to A+3 in *N. sarniensis* x 'Jill'; A-2 and A+5 in *N. sarniensis* x 'Rosea'; and A+8 to A+9 in *N. bowdenii* x 'Clone 63'. It therefore appears peak receptivity varies within and between species. Importantly, the duration of receptivity also varied within and between species. Receptivity was much shorter in *N. sarniensis* x 'Jill' (2 days) than in *N. sarniensis* x 'Rosea' (14 days). *N. bowdenii* x 'Clone 63' was intermediate, with a receptive period spanning eight days.

No seed was set prior to A-5 in any controlled pollinations in *N. sarniensis* x 'Rosea' or *N. bowdenii* x 'Clone 63'. This may be due to immaturity of the embryo sac. Pollination at A-5 would result in the pollen tube entering the ovule at A-3. When one considers that formation of the embryo sac in some florets has been found as late as A-4 (see Chapter 2) this is a possibility. The failure of crosses after A+12 is probably due to the onset of floral senescence prior to completion of the fertilisation process as number of pollen tubes in the style remains high at this late stage.

These patterns of receptivity have implications for plant breeders. Whilst the lengthy receptive period of *N. sarniensis* x 'Rosea' means pollen can be applied at any time from A-3, it also means stigmata must be covered during this time to prevent unwanted pollinations. Crosses using *N. sarniensis* x 'Jill' as a seed parent should be performed two or three days after anthesis. However, as it is possible for fertilisation to occur at anthesis and up to A+7, stigmata should be covered until this period has passed. Similarly, for maximum chance of success, crosses using *N. bowdenii* x 'Clone 63' should be performed from A+5, although the small likelihood of fertilisation from pollination prior to this period necessitates the covering of the stigma.

The variation found in cultivars of the same species suggests that the peak receptive period would need to be determined for each individual cultivar being used for crossing. Nevertheless, two generalisations can be inferred from the data. Firstly, although peak receptivity varies, there is a small chance of successful fertilisation from any crosses performed at or after anthesis. The end point of this receptive period varies with cultivar, but occurs prior to senescence of the floret due to the length of time required for the fertilisation process (three days). In *N. bowdenii* longevity of the floret means fertilisation can occur from crosses performed up to A+12. In *N. sarniensis* x 'Jill', senescence occurs earlier, therefore receptivity ceases earlier. During this period, stigmata must be covered to exclude unwanted pollination.

Secondly, crosses performed in the period from A+2 to A+5 had a reasonably high success rate in the three cultivars tested. Where pollen is limited and exact receptivity unknown, application during this period would have the greatest chance of success. If possible, repeat applications with fresh pollen at the beginning and end of this period would be desirable.

In the *Nerine* cultivars tested, morphological indicators, such as maximum ovary diameter, style length and anther dehiscence, do not consistently correlate to period of maximal receptivity as determined directly by seed set. In *N. sarniensis* x 'Jill' and x 'Rosea' anther dehiscence occurs within the receptive period but does not overlap with peak receptivity. This receptive period, however, extends beyond the availability of self pollen. In *N. bowdenii,* each of the second whorl of anthers open within the peak receptive period, yet the first whorl of anthers open prior to this time. Whilst anther dehiscence does not directly indicate receptivity, in all cultivars the stigma is in close proximity to the anthers during the receptive period, and at least some of the anthers dehisce during this time.

The *Nerine* stigma is dry and papillate, of type IIb(i) according to the classification of Heslop-Harrison and Shivanna (1977). Papillate, trilobate stigmata are commonly found in the Amaryllidaceae (Dahlgren and Clifford, 1982). In *Nerine*, spreading of the stigmatic papillae begins just before anthesis and proceeds gradually. Subsequently, and well after anthesis, the stigmatic lobes spread to expose the central transmitting channel. As with other morphological changes this does not appear to be directly related to receptivity. It does however, correlate with data on number of

pollen grains present on the stigma and number of pollen tubes found within the style. The increase in number of pollen grains adhering to the stigma with age is as expected due to the increased surface area and papillar topography. The very low number of pollen grains adhering to the stigma in pre-anthesis pollinations can be explained by the tightly packed stigmatic papillae not being amenable to pollen capture. Indeed an important function of stigmatic papillae is to increase capture area of the stigma (Heslop-Harrison and Shivanna, 1977) and this appears to be the case in *Nerine*. Stigmatic papillae also reduce the opportunity for interaction between the stigma and the pollen grain ensuring pollen surface materials cannot be distributed too far from individual grains (Heslop-Harrison and Shivanna, 1977). This would, presumably, lessen inhibitory effects of surface proteins from non-compatible pollen.

Maximum style length occurs within the peak seed set period of *N. sarniensis* x 'Rosea'. In this cultivar, style length reaches a maximum at approximately A+2 from which point, seed set is relatively high, yet it is also high at A-2. This suggests style length is not directly related to receptivity. In both *N. sarniensis* x 'Jill' and *N. bowdenii* x 'Clone 63', maximum style length occurs after peak receptivity. In *N. bowdenii*, the lengthy period of style elongation may be related to the late onset of peak receptivity in this species. However, a high percentage of successful crosses can occur up to six days prior to maximum length of the style being reached. The continuing growth of the style may therefore be an adaptive feature, in the presence of a long receptive period, to increase the likelihood initially of self pollination (when passing the anthers at *ca.* A+5) and open pollination (when extruded from the floret after A+7). Delayed stylar extension and protandry, as seen in the short-styled cultivars of *Nerine*, has also been observed in the Myrtaceae and is thought to encourage outcrossing (Moncur and Boland, 1989).

The number of pollen tubes at the top and base of the style increases with time after anthesis. This occurred in both *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea'. Pollen grains were seen germinating on the stigma following crosses performed prior to anthesis, in both cultivars. However, pollen tubes were not found in significant numbers in the style until much later. In *N. bowdenii* x 'Clone 63', pollen tubes are first observed at the base of the style at A+6 generally in extremely low numbers (15 or less). This corresponds with a late period of successful seed set. Although seed set was relatively high after A+5 (peaking at A+8 and A+9), maximal numbers of pollen tubes do not occur until after this period. In *N. sarniensis* x 'Rosea', pollen tubes have been found in the base of the style from A-2. This is

much earlier than in *N. bowdenii*, and corresponds with the earlier seed set in this cultivar. As seen in *N. bowdenii* x 'Clone 63', the overall pattern once again increases with age and does not reflect the pattern of actual seed set.

Pollen tube number, either at the base or at the top of the style, does not appear to indicate likelihood of successful seed set in these cultivars. However, the lack of penetration of germinated pollen grains at early stages may be attributable to the tightly packed papillae. The opening of the papillae, increasing stigmatic surface area and exposing the channel, would explain the large numbers of pollen grains found on the stigma and in the style from A+9 to A+11.

In controlled pollinations performed throughout the life of the floret, a general reduction in the number of pollen tubes occurs with progress down the pistil. This reduction may be due to competition for space to penetrate the stigma or competition between fastest growing pollen tubes to reach the ovules (O'Brien, 1996). However, in *Nerine*, the number of pollen tubes in the style greatly increases with age. This would be an expected consequence of the greater numbers of pollen grains found once the stigmatic lobes and papillae are fully open, together with the progressive exposure of the transmitting channel. As this opening occurs towards the end of the receptive period in both cultivars, it may be a final strategy to ensure fertilisation. At this final stage, pollen tube competition, although still occurring does not cause such a severe reduction in numbers of pollen tubes reaching the ovules. A similar delayed opening of the stigma has been reported in the closely related genus *Crinum* (Howell and Prakash, 1990).

Esterase is an important component of stigma surface proteins in both wet and dry (as in *Nerine*) stigma surfaces and its secretion is thought to be related to receptivity (Heslop-Harrison *et al.*, 1975; Shivanna and Rangaswarmy, 1992). Localisation of esterase has been used successfully to identify both receptive surfaces (Mattson *et al.*, 1974; Kenrick and Knox, 1981) and to indicate when stigma surfaces are receptive for pollen germination (Bernhardt *et al.*, 1980; Sedgley *et al.*, 1985; Collins and Spice, 1986). However, whilst esterase may be an indication of stigmatic receptivity and consequent amenability to pollen germination, it cannot be assumed stigmatic receptivity is indicative of ovule receptivity. In *Cucurbita pepo*, for example, stigmatic and ovule receptivity are not synchronised (Nepi and Pacini, 1993).

In the cultivars of *Nerine* studied, stigmatic esterase activity follows a similar pattern exhibiting a general increase with age of the floret. In *N. sarniensis* x 'Rosea', the pattern of esterase activity reaches a maximum from A+6 to A+11, occurring after the period of maximal seed set. Similarly in *N. bowdenii* x 'Clone 63', peak esterase activity (A+9 to A+12) does not correlate with maximum seed set. The slightly earlier onset of high esterase reading in *N. sarniensis* x 'Rosea' may be due to the reduced longevity of the floret.

The similarity in esterase activity patterns between cultivars and absence of correlation with actual seed set indicates it is not a good measure of ovule receptivity. However, it does correlate with the progressive opening of the stigmatic lobes and number of pollen tubes in the pistil and as such appears to be a good indicator of stigmatic receptivity. Nevertheless the possibility that esterase activity is a reflection of stigmatic age, rather than a function of receptivity cannot be ruled out. It is unlikely that a high esterase activity at A+11 and A+12 is indicative of receptivity. Pollinations occurring at this late stage may not achieve fertilisation, which takes approximately three days, before floret senescence occurs at A+11 to A+14. Use of esterase to mark receptivity has also been questioned by O'Brien (1996) who proposed that esterase activity may give an over-estimation of receptivity by staining positively when receptivity is only beginning, prior to functional receptivity being achieved. In *Nerine*, this may also be true, as some esterase activity was detected on pre-anthesis stigmata at stages where seed was not successfully set.

Appearance of callose in pistil tissues may be associated with senescence, marking the end of the receptive period, and as such has been suggested as a useful phenotypic bioassay for ovule receptivity (Dumas and Knox, 1983). Loss of viability for pollen tubes is indicated by appearance of callose in some species including *Prunus*, *Malus* and *Rhododendron* (for review see Dumas and Knox, 1983). In *Nerine*, callose was noted in ovules in late stages of receptivity. However as this coincided with the presence of large numbers of pollen tubes, also known to produce callose (Heslop-Harrison, 1975), it could not be confirmed as being maternal in origin. In order to positively identify the callose as originating from pistil tissues, unpollinated styles would need to be examined for callose using the aniline blue fluorescence method. Nevertheless, at least in *N. sarniensis*, the receptive period, as directly measured from seed set, extended for almost the entire life of the floret, with high seed yields early in the receptive period. It therefore appears

unlikely that callose, as an indicator of senescence, would be a good indicator of receptivity in this species.

The results from this study show that the two species of *Nerine* have quite different patterns of seed set. They also indicate no indirect assessment of fertility used in this study correlates with the period of maximum seed set, directly measured, in either *N. bowdenii* x 'Clone 63' or *N. sarniensis* x 'Rosea'. As stigmatic morphology, number of pollen tubes in the pistil and esterase activity are relatively coordinated, they may, in fact, signify stigmatic receptivity. However if this is the case, stigmatic receptivity is not synchronised with ovular receptivity in *Nerine*. This extension of stigmatic receptivity beyond peak ovular receptivity, but within the period in which seed may be set, may provide a final opportunity for florets which had yet to be pollinated by opening up the stigma and allowing large numbers of pollen grains to enter the style and correspondingly large numbers of tubes to reach the ovary. No extensive study has been found that has compared direct seed set with a number of indirect methods, although O'Brien (1996) has found a discrepancy between receptivity as marked by esterase and pollen germination in *Chamelaucium uncinatum*. Similarly, there are cases where stigmatic and ovular receptivity are not synchronised (Nepi and Pacini, 1993).

This varying pattern of seed set and lack of correlation with morphological changes (Table 4.7) has implications for breeders of *Nerine*: receptive periods for all cultivars would need to be determined from direct measurement of seed set. Opening of the stigmatic lobes and maximum ovary diameter occur at approximately the same time in both cultivars with style elongation and anther dehiscence showing some contrast between varieties. This contrast means that once seed set has been determined for a particular cultivar, it would be possible to use physical markers such as style length or anther dehiscence as a reference point. Furthermore, although these physical markers do not directly delineate receptivity they may be related to receptivity and seed set.

Cultivar	Stigmatic opening	Style length	Ovary diameter	Anther dehiscence	Esterase activity	Pollen tubes in style	Seed set
N. bowdenii x 'Clone 63'	A+7	A+11	A+4	A+2 to A+10	A+8 to A+12	A+9 to A+11	A+5 to A+12
N. sarniensis x 'Rosea'	A+7	A+3	A+3	A+1 to A+4	A+6 to A+11	A+9 to A+11	A-3 to A+10

Table 4.7: Summary of the peak periods of receptivity as determined by indirect and direct methods for *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea'.

The relationship between morphology and receptivity may be a result of adaptation to a particular mechanism of pollination. Both *N. sarniensis* and *N. bowdenii* exhibit mechanisms that partially separate male and female phases of the flower. In *N. bowdenii*, although viable pollen (less than two days old) is available almost entirely throughout the receptive period, the stigma becomes spatially separated from the anthers by stylar elongation, a form of herkogamy (Webb and Lloyd, 1986). Herkogamy is thought to be involved in the prevention of self-fertilisation or reduction of self interference (Wilson and Thomson, 1991). In *N. sarniensis*, although the style extends slightly, the separation is not spatial, but temporal, (*i.e.* dichogamous, [Lloyd and Webb, 1986]) with all anthers having dehisced early in the receptive period.

Pollination in *Nerine* may occur by a number of different mechanisms. The cultivars used in this study were all self-fertile, all setting seed from controlled self pollinations. It is likely that self pollination is a significant occurrence in the field as both *N. bowdenii* and *N. sarniensis* show similar rates of seed set in open pollinated plants, and in plants that have been bagged to prevent non-self pollination. Self pollination may be facilitated when elongation of the style brings the stigma very close to the dehiscing anthers in short-styled species. In exsert- and level- styled species, the first anther dehisces 1-2 days after anthesis, again bringing the stigma in close proximity to fresh pollen. Mechanical movement of the florets, such as that by wind, can result in pollen being deposited, particularly in the level- and short-styled species.

As the style elongates it protrudes from the floret, exposing the stigma to air-borne pollen and to insect pollinators. Insect pollinators observed include bees, butterflies and small spiders (N. Brown, pers. obs.). Spiders have been seen to weave webs across the floret, increasing the likelihood of self pollination, especially in the short-styled species. Elongation of the style can be an adaptation to promote or

alternatively reduce opportunities for self deposition of pollen; depending upon the original position of the stigma. Exsertion of the stigma has been shown to have an effect on pollen deposition by Thomson and Stratton (1985), working on *Erythronium* flowers. In *Erythronium*, shorter-styled flowers had a larger proportion of self pollen deposited than did longer-styled flowers. This is probably also the case in *Nerine*, where shorter-styled varieties show a greater percentage of self pollination than longer-styled varieties in bagged plants.

In the short-styled *N. bowdenii*, elongation of the style past the anthers coincides with the time of high receptivity (as determined by seed set). This species also exhibits delayed anther dehiscence; the final anther opening around the time of maximal receptivity. This suggests that the species may be adapted towards self pollination. This could also account for the difference in open pollination rates of the short-styled *N. bowdenii* as opposed to the longer styled *N. sarniensis*. In fact, from open pollination results, it appears that the short style morph is advantageous for successful pollination with all three short-styled species; *N. bowdenii*, *N. flexuosa* and *N. masonorum* have higher rates of seed set than *N. sarniensis* cultivars in field conditions.

The short-styled *N. masonorum*, however, does not fit the above scenario. This may be due to one or a combination of factors. As plants were grown in plastic tunnels, they may not have been exposed to wind and associated movement of the florets to the extent of field grown plants. This may have resulted in less self deposition of pollen than if grown in the field. Additionally, *N. masonorum* started flowering much earlier than other varieties, and as such, number of pollinators attracted to the blooms in the tunnel would have been low. At the peak of the flowering season, when most of the *N. masonorum* bulbs were either in flower or in bud, the number of seeds per floret increased. Similarly, the number of plants with seed increased at the end of the flowering season, which coincided with the flowering season of the majority of other cultivars in the tunnel, and presumably a greater number of pollinators attracted to the inflorescences. This suggests that pollinators may be important, at least in this species.

The reduced rate of seed set from self pollination in bagged plants of *N. flexuosa* 'alba', compared to the open pollinated controls may well be due to the exclusion of pollinators or the restriction of pollen flow by the reduction of floret movement. This reduction in fertility is unlikely to be due to any form of self incompatibility, as any

such mechanism would be expected to act also on pollen from surrounding plants, which are clones of a single parent bulb. A possible explanation may be that fertilisation does not usually occur from the same floret. This does not mean that self pollination would not occur in the field, but the self pollen may originate from the anther of another floret on the same inflorescence. The bagging, by restricting movement and pollinator access, may reduce the pollen flow from floret to floret, accounting for the observed reduction in seed set. In this variety it is possible spatial separation of the pistil and anthers after stylar elongation, and/or temporal separation of the male/female reproductive phases may be occurring and therefore lessening the opportunity for within floret self pollination. Controlled crossing to determine receptive period in this variety would assist in interpreting these observations.

The results from hand pollination of *N. sarniensis* x 'Rosea' also supports the theory that short-style cultivars are better adapted to ensure maximum seed yield. Certainly, *N. sarniensis* x 'Rosea', has the physiological potential to bear many seeds per inflorescence, with up to 19 being formed when hand pollinated. The smaller number of seeds borne per plant in an open pollinated situation therefore appears to be due to physical rather than biological constraints, and could be due to limited availability of pollen. This in turn could be explained, at least in part, by the shorter period of self pollen availability, and the position of the stigma being above dehiscing anthers making self deposition difficult in the absence of pollinators. The higher open pollination rate in field grown *N. sarniensis* x 'Rosea', in comparison to those grown in the tunnel, suggests pollination may occur more effectively in the field. This may be due to a greater number of available pollinators, again suggesting the importance of pollinators. Alternatively, greater movement of the florets through exposure to prevailing weather conditions may facilitate self pollination, and hence increase seed set.

It is thought herkogamy, of which the stylar extension exhibited by the short-styled *Nerine* cultivars is a form, may have the effect of reducing self pollination by minimising the chance of a pollinator brushing pollen directly from anther to stigma (Webb and Lloyd, 1986). However, it by no means eliminates the chance of self pollination as has been shown in *Leptospermum*, where herkogamy in combination with protandry did not prevent self fertilisation (O'Brien and Calder, 1993). In *N. bowdenii* this spatial separation, appears to have a negligible effect on self fertilisation, which is evident from the result of seed set in bagged plants. This may be due to the late occurrence of the spatial separation by which time self pollination

could already been effected in many florets. It may also be due to the effectively hermaphrodite nature of *N. bowdenii*, with male and female phases overlapping, and spatial separation not able to preclude selfing.

In some members of *Narcissus*, style growth in long-styled plants proceeds during anther dehiscence so stigmata are at the same level of the anthers during early male function, but project above them during stigma receptivity (Barrett *et al.*, 1996). This pattern of development is similar to that seen in *N. sarniensis* and has been interpreted by Barrett and co-workers (1996) as a mechanism to reduce pollen-stigma interference. In this species, the male phase occurs early in the life of the floret (A+1 to A+6) with the female phase extending beyond A+12.

The partial dichogamy, as seen in *N. sarniensis*, along with minimal style elongation seem to be more effective in preventing self pollination. A shift in timing of stigma receptivity in relation to anther dehiscence, is thought to be a temporal mechanism, which increases outcrossing rates in self compatible species (Palmer *et al.*, 1989). Yet, as with herkogamy, it does not eliminate self pollination and fertilisation; in this case it is due to partial overlap of male and female phases within the same floret.

Whilst within flower self pollination may be lessened by spatial or temporal separation, selfing is not precluded. In particular, the relationship with other florets in the individual needs to be considered (Snow *et al.*, 1996). According to Snow and co-workers (1996), protogyny and protandry are seldom synchronised within an inflorescence, so self pollen can be transferred from other florets on the same plant. This asynchronous flowering occurs in *Nerine* so viable pollen is available within the inflorescence. This would mean some florets, depending on position in the flowering sequence, would have access to fresh pollen from the same plant for the whole of the receptive period despite the individual floret exhibiting partial dichogamy. However, some method of pollen transfer between florets would be required to effect pollination. In *Allium*, a protandrous genus, such asynchronous flowering has the net effect of flowers not being protandrous (Currah and Ockenden, 1978). In *Nerine*, the smaller number of florets per inflorescence and the greater distances between florets, would not totally overcome protandry as is seen in *Allium*, especially in the absence of pollinators.

Floral longevity (from anthesis to floral senescence) is important in reproductive biology, by influencing the number of pollinator visits and consequently the amount

as well as diversity of pollen received (Ashman and Schoen, 1996). The amount of pollen able to be disseminated can also increase providing the pollen remains viable. In the case of *Nerine*, pollen from an individual anther does not retain viability for longer than four days, yet the asynchronous anther dehiscence within an individual flower has a net result of viable pollen being available for a lengthy period, and in the case of *N. bowdenii* almost the entire life of the floret. However, increasing longevity drains resources of the plant and needs to be balanced with these benefits.

Asynchrony of flowering within an individual inflorescence is thought to attract pollinators (Rathcke and Lacey, 1985), but may also reflect uncertainty of pollination (Travis, 1984). Asynchrony in *Nerine*, may be due to this, as individual floret morphology and physiology, the extending of the style out of the flower in the latter stages of receptivity, may also reflect this uncertainty by providing a 'last chance' for pollination.

Position in the opening sequence of an individual floret does have a bearing on fertility as a seed parent, both in open pollinations and self pollinations in the presence of unlimited pollen. These findings support Horovitz (1978) who argues that outcrossing hermaphrodite flowers are not as effective as male and female parents. Non-uniformity of seed set within an inflorescence is common and may be related to temporal or spatial factors dictating access to pollen, or following fertilisation, resources for seed development (Diggle, 1995). In *N. bowdenii*, the failure of the first floret to set seed may be indicative of embryo sac immaturity or the direction of resources into other florets in the inflorescence. Similarly, the reduced seed yield of late-opening florets may be due to a drain of resources available to the senescing inflorescence.

In *N. bowdenii*, chances of self pollen contacting the stigma by accidental movement or by pollinator transferral are reduced. Additionally, in *N. sarniensis*, protandry prevents the style from being covered with self pollen in these latter stages of receptivity making it available for non-self pollen. These strategies may be a last resort to expose unpollinated pistils to non-self pollen nearing the end of the receptive period. Whether or not physiological changes also favouring outcrossing occur late in the receptive period remain to be determined by controlled crossing for the duration of the receptive period using non-self pollen.

4.5 Conclusion

In *Nerine* the fertilisation process from pollen germination to entry of the pollen tube into the micropyle takes approximately three days. The onset, duration and period of maximal pistil receptivity, as determined by actual seed set, varies within and between species and does not correlate with indirect measures. Pollinations performed 2-5 days after anthesis have a relatively high success rate in the cultivars tested, however, fertilisation can occur from pollinations as late as 12 days after anthesis, so in controlled crossing stigmata should be covered until this time to prevent unwanted open pollination.

The length of the style at anthesis, relative to anther position, can be in one of three positions in *Nerine* (below, level or exsert). In all cases, elongation of the style occurs post-anthesis, with maximal elongation occurring in the short-styled cultivars. These short-style morphs have a greater rate of seed set from both open and self-pollinations.

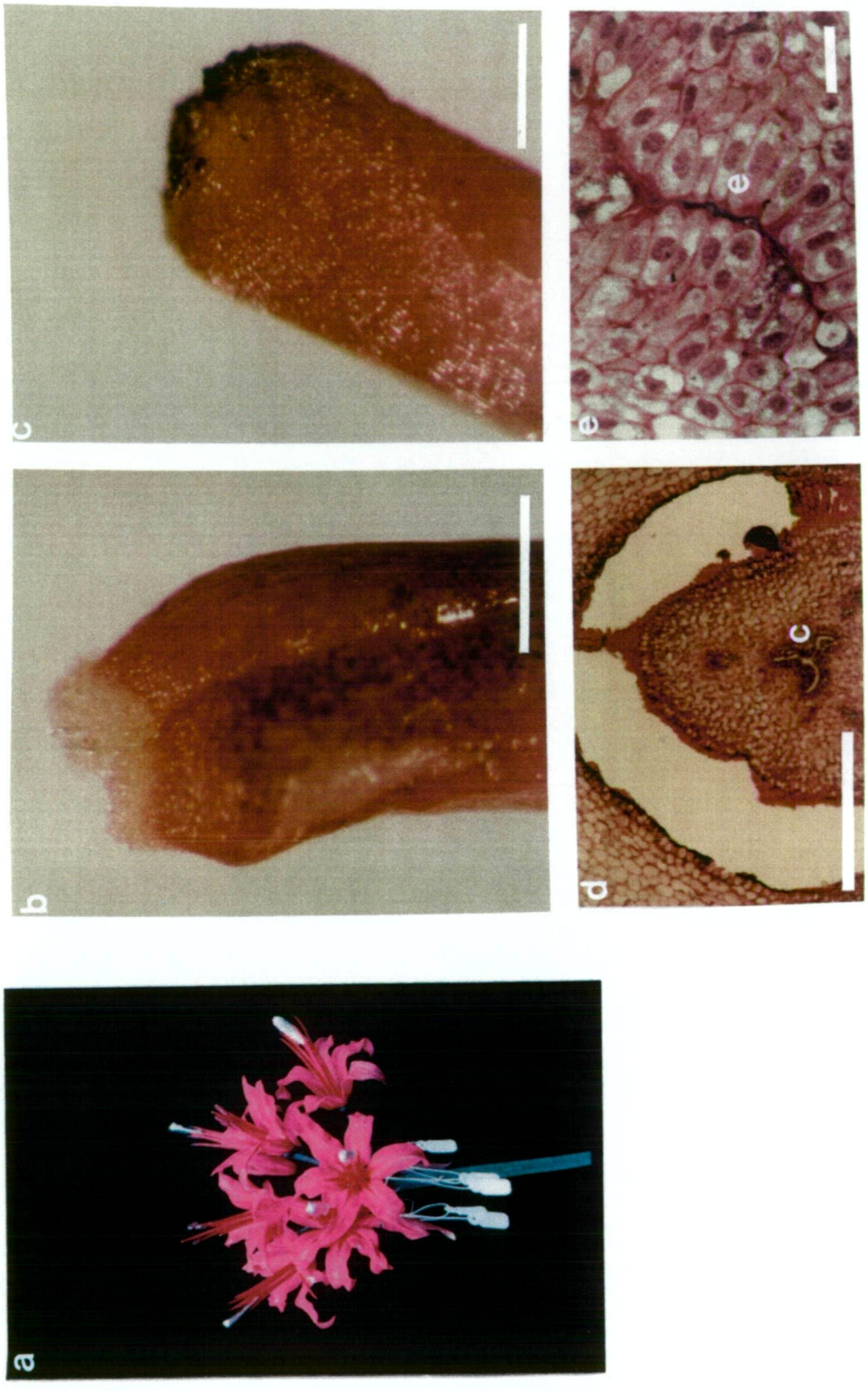
Plate 4.1a Inflorescence of *N. sarniensis* x 'Cherry Ripe' showing florets tagged after controlled crossing.

Plate 4.1b Detection of esterase on stigmata - control stigma. Scale bar = 1mm.

Plate 4.1c Detection of esterase on stigmata - stained stigma. Scale bar = 1mm.

Plate 4.1d Transverse section of *Nerine* style base through tri-radiate stylar canal (c). Scale bar = 500μm. (Stain: Toluidine blue.)

Plate 4.1e High power micrograph of elongated cells (e) lining stylar canal. Scale bar = 50μm. (Stain: Toluidine blue.)


c
e
b
d
a

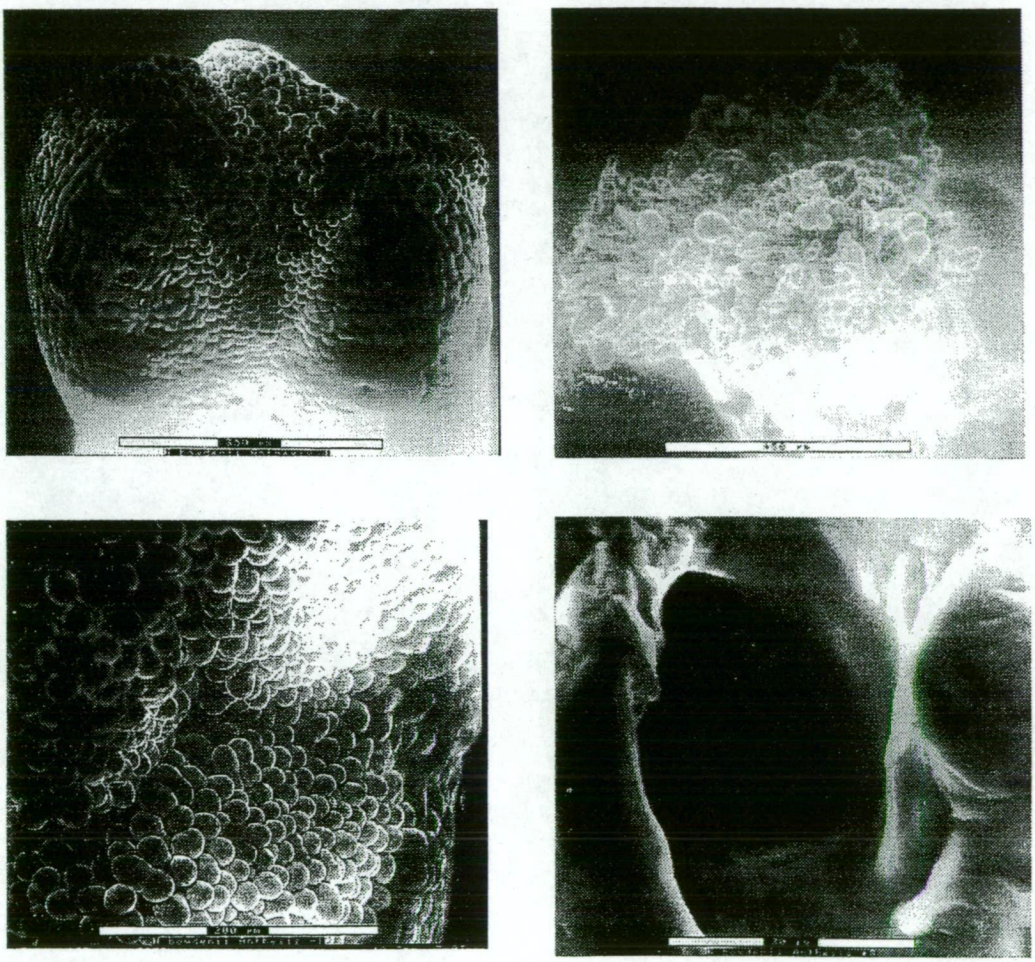


Plate 4.2a Micrograph (esem) of *N. bowdenii* x 'Clone 63' stigma at A-4. Note closed stigmatic lobes and tightly packed papillae.

Plate 4.2a Micrograph (esem) of *N. bowdenii* x 'Clone 63' stigma at A+1.

Plate 4.2c Micrograph (esem) of *N. bowdenii* x 'Clone 63' stigma at A+6. Note spreading of stigmatic lobes and erect papillae.

Plate 4.2d Micrograph (esem) of *N. bowdenii* x 'Clone 63' stigma at A+6, showing entrance to the stylar canal.

Plate 4.3a Floret of *N. sarniensis* x 'Fothergillii major' (an exsert-styled cultivar), showing relative position of anthers and style at anthesis. Scale bar = 0.5cm.

Plate 4.3b Floret of *N. bowdenii* x 'Winter Cheer' (a short-styled cultivar), showing relative position of anthers and style at anthesis. Scale bar = 0.5cm.

Plate 4.3c Position of the style relative to the anthers in *N. bowdenii* x 'Clone 63' (a short-styled cultivar), at (L to R); A-6, A-4, A-3, A, A+10. Scale bar = 2cm.

Plate 4.3d Position of the style relative to the anthers in *N. sarniensis* x 'Corusca' (an exsert-styled cultivar), at (L to R); A-6, A-5, A-4, A-3, A-2, A-1, A, A+2. Scale bar = 2cm.

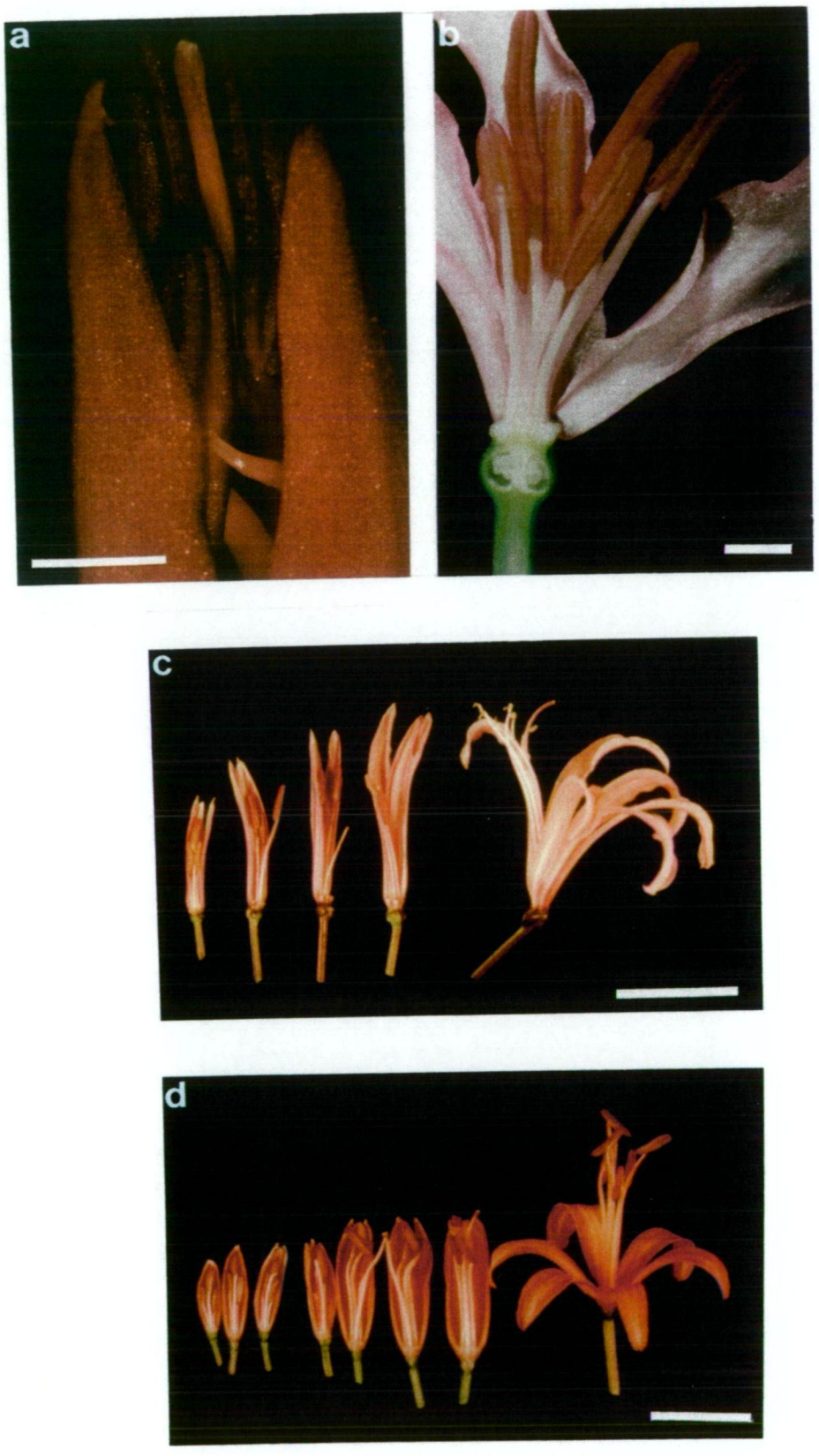

a
b
c
d

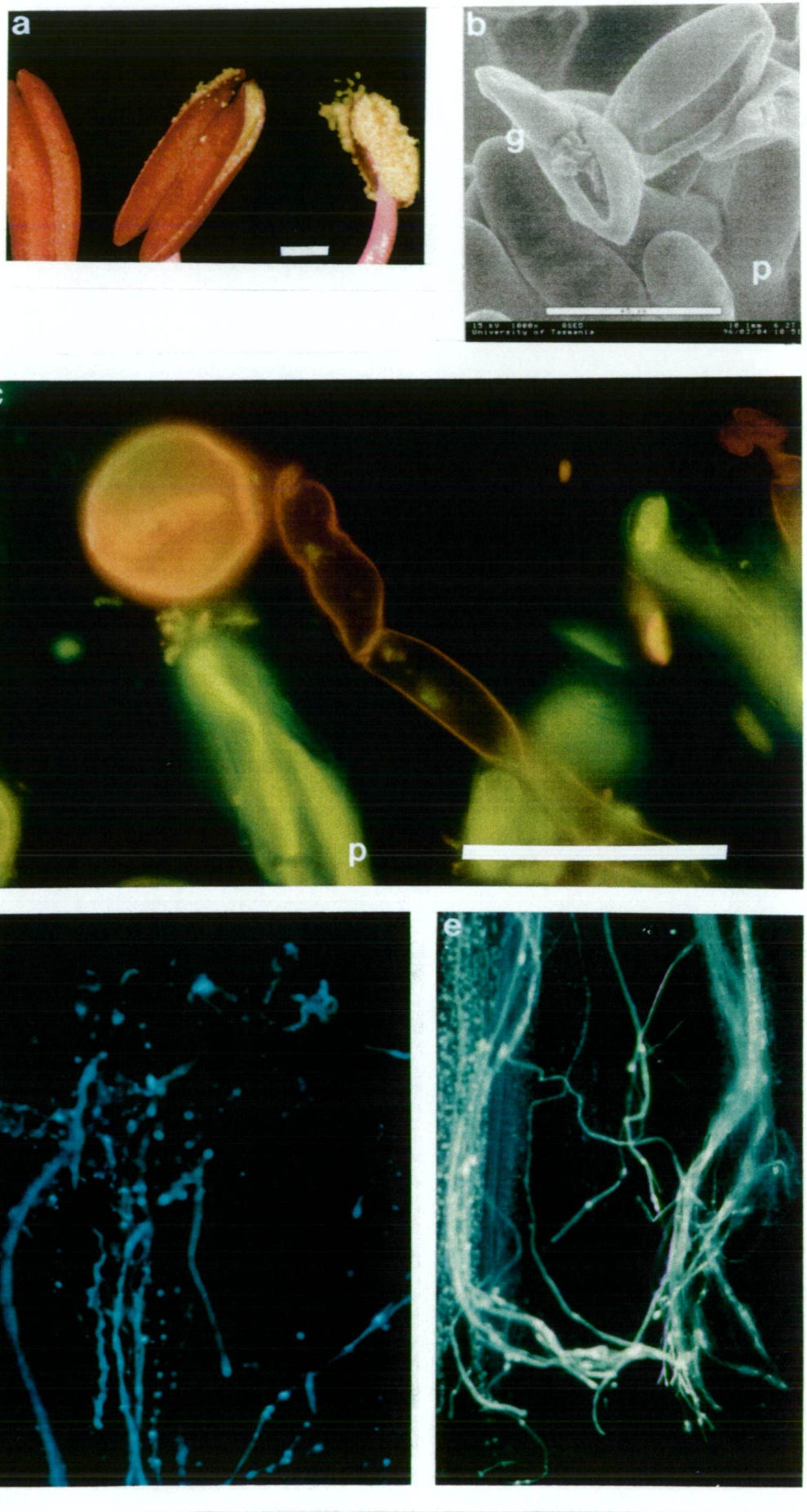
Plate 4.4a Early and late stages of anther dehiscence in *N. sarniensis* x 'Rosea'. Note mass of pollen grains after dehiscence. Scale bar = 1mm.

Plate 4.4b Micrograph (esem) of pollen grains (g) germinating between stigmatic papillae (p).

Plate 4.4c Pollen grains germinating between stigmatic papillae. Scale bar = 100μm. (Stain: Acridine orange.)

Plate 4.4d Pollen tubes penetrating stigma/style barrier. (Squash) (Stain: Aniline blue.)

Plate 4.4e Pollen tubes at the base of the style. (Squash) (Stain: Aniline blue.)


a
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g
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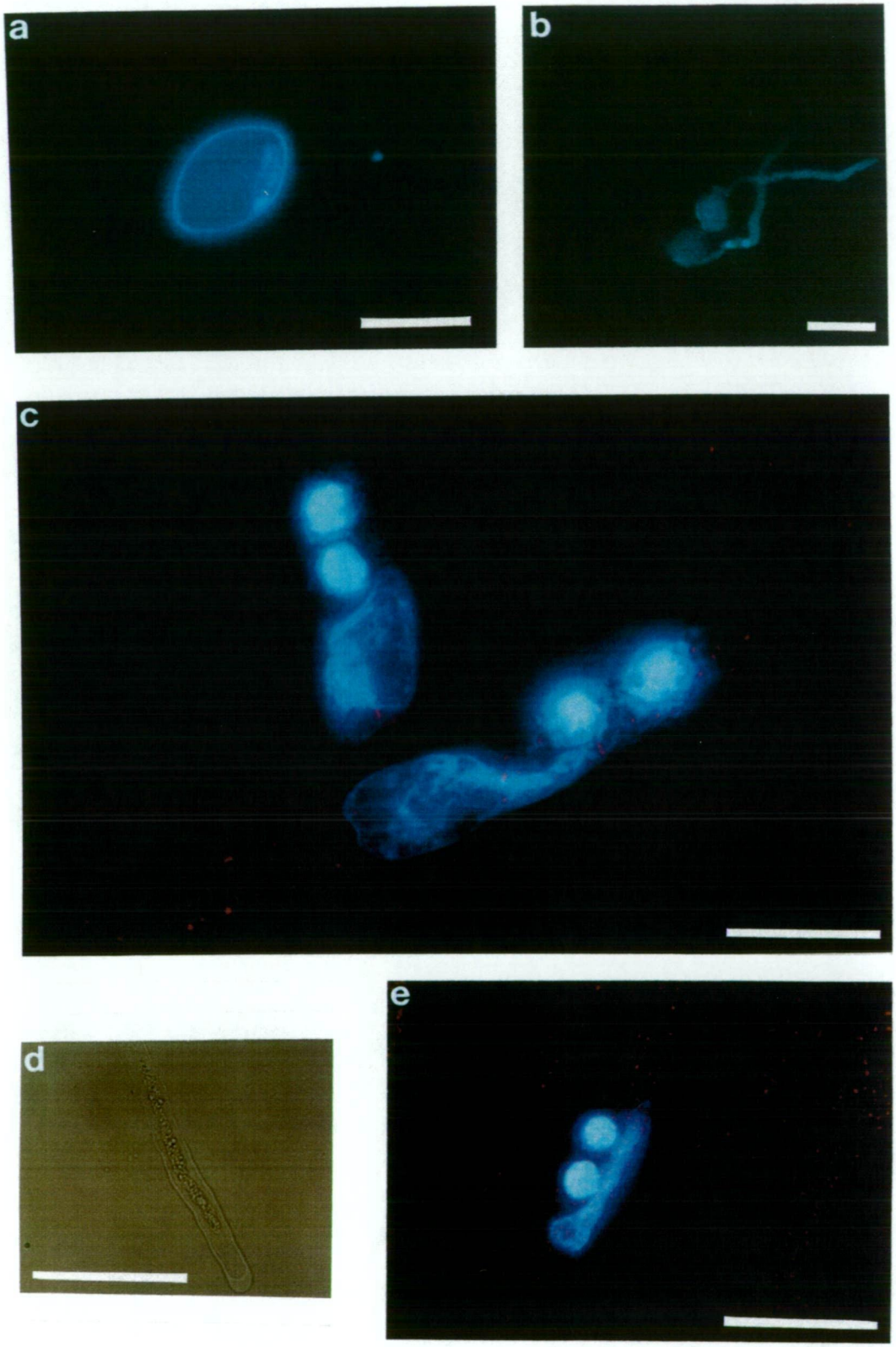
Plate 4.5a Mature binucleate pollen grain. Scale bar = 50μm. (Stain: Hoechst.)

Plate 4.5b Sperm cells and tube nucleus entering the pollen tube. Scale bar = 100μm. (Stain: Hoechst.)

Plate 4.5c The male germ unit of *Nerine*. The densely packed nuclei of the sperm cells contrasts with the dispersed tube nucleus. Scale bar = 50μm. (Stain: Hoechst.)

Plate 4.5d The growing tip at the distal end of the pollen tube. Scale bar = 100μm. (Unstained.)

Plate 4.5e Sperm cells adjacent to the elongated and flattened pollen tube nucleus. Scale bar = 100μm. (Stain: Hoechst.)


a
b
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Cultivar	A-5	A-4	A-3	A-2	A-1	A+0	A+1	A+2	A+3	A+4	A+5	A+6	A+7	A+8	A+9	A+10	A+11	A+12
N. bowdenii x																		
'Clone 63'		√		√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
'Pink Jewel'								√										
N. flexuosa 'alba'					√	√	√	√	√									
N. sarniensis x																		
'Angela Limerick'						√	√	√			√							
'Brahms'						√	√											
'Captain D. Cook'						√							√					
'Caroline'				√		√	√							√				
'Chorister'					√	√	√	√	√	√								
'Cuckfield'					√		√	√			√							
'Cynthia Chance'						√	√	√										
'Donna'				√			√											
'Enchantress'				√		√			√									
'Fothergillii major'				√	√													
'Fred Dank'							√			√								
'Gold Dust'				√		√	√	√										
'Indian Orange'						√				√								
'Jean O'Neill'						√			√	√								
'Jill'					√	√		√	√									
'Kenilworth'		√		√					√									
'Killi'				√		√												
'Mrs Bromley'					√	√		√										
'Mrs Cooper'				√	√	√												
'Pink Fairy'				√		√	√	√					√					
'Redhead'					√			√										
'Rosea'	√		√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
'Salmon Supreme'						√	√											√
'Sunset Falls'						√	√	√									√	√
'Xanthia'				√		√		√										

Table A.4.1 : Time of pollination for successful crosses (includes self and out crosses). For further detail see Appendix B: Breeding Records.
√ = successful cross recorded.

Nerine species/cultivar	% Plants with seed*	% Florets with seed*	Average seeds per seeded floret
N. bowdenii	100	77	4
N. bowdenii x 'Clone 63'	95	60	2.6
N. bowdenii x 'Winter Cheer'	0	0	-
N. filamentosa	0	0	-
N. flexuosa 'alba'	100	63	2
N. masonorum	53	16	1.4
N. sarniensis x 'Corusca'	100	35	2
N. sarniensis x 'Jill'	33	7	1.7
N. sarniensis x 'Rosea'	62	15	1.3
N. sarniensis x 'Fothergillii major'	4	4	1
N. undulata x 'Roseo-crispa'	21	4	1
Nerine x 'Ancilla'	0	0	-
Nerine x 'Cameo Beauty'	0	0	-
Nerine x 'Cranfield'	0	0	-
Nerine x 'Elvira'	0	0	-
Nerine x 'Gladys Dettman'	0	0	-
Nerine x 'Salmonea'	0	0	-
N. sarniensis x 'Cuckfield'	-	-	1.7
N. sarniensis x 'Cynthia Chance'	-	-	4.2
N. sarniensis x 'Early Snow'	-	-	3
N. sarniensis x 'Eve'	-	-	1.3
N. sarniensis x 'Fred Danks'	-	-	3
N. sarniensis x 'Gold Dust'	-	-	2.7
N. sarniensis x 'Indian Orange'	-	-	1
N. sarniensis x 'Kenilworth'	-	-	1
N. sarniensis x 'Mrs Bromley'	-	-	1.7
N. sarniensis x 'Pink Fairy'	-	-	2
N. sarniensis x 'Pink Opal'	-	-	1
N. sarniensis x 'Rosamund Elwes'	-	-	1.8
N. sarniensis x 'Shelagh Mulholland'	-	-	1.3

Table A.4.2: Summary of open pollination data 1995-1998. For full details refer to Table A.4.3. (* More than four plants were available for observation).

TAXON	Year	Plants	Florets	Seeds	Florets OP	Plants OP	Seed
Nerine flexuosa 'alba'	95	1	3	2	2	1	√
	96	4	36	21	14	4	√
	97	17	160	207	100	17	√
N. filamentosa	97	30	165	0	0	0	X
N. masonorum	96	19	121	0	0	0	√
N. undulata	97	12	109	45	33	11	√
N undulata x 'Roseo-crispa'	96	5	42	0	0	0	√
Nerine x							
Afterglow	96	1	2	0	0	0	X
	98	1	5	0	0	0	X
Ancilla	96	2	4	0	0	0	X
	97	2	19	0	0	0	X
Angela Limerick	97	1	1	0	0	0	√
Aurora	96	1	2	0	0	0	X
	97	1	2	0	0	0	X
Bagdad	96	1	10	0	0	0	X
Brahms	95	1	10	20	7	1	√
	96	1	2	0	0	0	X
Cameo Beauty	97	3	13	0	0	0	X
Canasta	96	1	4	0	0	0	√
	97	1	4	0	0	0	√
Captain Dunne Cook	96	2	11	0	0	0	√
	97	1	4	0	0	0	√
Caroline	96	1	2	0	0	0	√
Cherry Ripe	96	1	1	0	0	0	X
	97	1	3	0	0	0	X
Chorister	96	1	5	1	1	1	√
	97	1	5	0	0	0	√
Corusca	96	2	7	4	2	3	√
	97	2	19	10	2	6	√
Cranfield	96	2	8	0	0	0	X
	97	2	19	0	0	0	X
Cuckfield	97	1	5	2	1	2	√
	98	1	1	1	1	1	√
Curiosity	96	1	6	0	0	0	X
	97	1	4	0	0	0	X
Cynthia Chance	98	1	4	17	4	1	√
Donna	95	1	6	1	1	1	√
	96	1	5	0	0	0	√
Early Snow	95	1	6	3	1	1	√
	97	1	7	0	0	0	√
Elvira	96	2	18	0	0	0	X
	97	2	12	0	0	0	X
Enchantress	97	1	2	0	0	0	√
Erubescens	96	1	8	5	1	2	√
Eve	96	1	7	0	0	0	√
Evelyn Humphries	97	1	4	0	0	0	X
Flame	96	1	3	0	0	0	√(TC)
Flame Brilliant	97	1	8	0	0	0	X
Fletcherii	98	1	4	0	0	0	X
Fothergillii	96	1	5	0	0	0	X
Fothergillii Major	95	13	140	0	0	0	√
	96	8	92	1	1	1	√
	97	3	22	0	0	0	√
Fred Dank	96	1	5	2	1	1	√
Galaxy	96	1	4	0	0	0	X

TAXON	Year	Plants	Florets	Seeds	Florets OP	Plants OP	Seed
Gladys Dettman	96	2	12	0	0	0	X
	97	2	8	0	0	0	X
Gilbert Errey	96	2	12	0	0	0	X
Gold Dust	96	1	3	8	3	1	√
Guy Fawkes	96	1	10	0	0	0	X
Hera	98	1	5	0	0	0	X
Imp	95	1	8	0	0	0	√
	96	1	3	0	0	0	√
Indian Orange	96	1	3	0	0	0	√
Jean O'Neill	96	2	8	2	2	2	√
Jenny Wren	96	1	3	0	0	0	X
Jill	96	57	499	46	30	16	√
	97	42	368	52	29	18	√
Kenilworth	97	1	6	4	4	1	√
Killi	96	1	3	0	0	0	√
Lady Lucy	95	1	11	1	1	1	√
Lucinda	97	1	3	0	0	0	X
Mansellii	96	1	7	0	0	0	X
Mother of Pearl	96	1	1	0	0	0	X
Mrs Cooper	96	1	6	0	0	0	√
Novelty	97	1	1	1	1	1	√
Old Rose	96	2	6	0	0	0	X
	97	2	13	0	0	0	X
Pink Fairy	96	1	4	2	1	1	√
Pink Opal	96	1	10	1	1	1	√
	97	1	4	1	1	1	√
Redhead	97	1	5	0	0	0	√
Rosea (Aust)	95	5	33	13	7	4	√
Rosita	97	1	5	0	0	0	X
Rose Princess	95	1	6	0	0	0	X
Salmon Supreme	96	1	1	0	0	0	√
Salmonea	95	3	29	0	0	0	X
	96	9	136	0	0	0	X
	97	7	111	0	0	0	X
Snow Maiden	96	1	1	0	0	0	X
Splendens (A)	96	1	3	0	0	0	√
Sunset Falls	96	1	1	0	0	0	√
Western Sunset	96	1	3	0	0	0	√
White Dove	96	1	1	0	0	0	X
Winter Cheer	96	6	54	0	0	0	X
	97	8	66	0	0	0	
Xanthia	95	1	6	0	0	0	√
	96	1	2	0	0	0	

Table A.4.3: Open pollination (OP) data for Channel Bulbs *Nerine* collection 1995-1998.

Chapter Five

Embryogenesis and seed germination in *Nerine.*

5.1 Introduction

The ovary of *Nerine* is inferior, possessing three locules with a varying number of anatropous ovules present in each locule (Dahlgren and Clifford, 1982). The ovules have a single, multi-layered integument with obvious stomata (Dahlgren *et al.*, 1985). The embryo sac is elliptical in shape, and is mature prior to anthesis of each individual floret (see Chapter 2).

In *N. bowdenii* and *N. sarniensis*, the process from pollen germination to pollen tube penetration of the micropyle takes approximately three days and fertilisation may result from pollinations occurring from anthesis of a floret up to 14 days after anthesis (see Chapter 4). Following successful fertilisation, seeds increase greatly in size and eventually burst through the ovary/fruit wall. The number of seeds produced per capsule varies within and between species, with 1-3 seeds commonly produced. However, seed numbers as high as 25 have been formed in *N. sarniensis* cultivars (see Chapter 4).

Nerine seeds are large, fleshy, globose to ovoid, with a stomatose epidermis and are water-rich; *N. sarniensis* seeds have a water content of 73.8% (Isaac and McGillivray, 1965). This is unusual as most angiosperm seeds are desiccated at maturity, containing only 5-15% water (Bewley and Black, 1994). Furthermore, the seeds are unusual as they possess stomata and contain chlorophyll in the integument. These features are rarely found in angiosperm seeds (Maheshwari, 1950). The presence of chlorophyll in seeds of *Nerine*, as well as the closely related

Brunsvigia and *Amaryllis*, was first identified by Hofmeister (1861, cited in Maheshwari, 1950). Stomata were first observed in the seed coat of *N. sarniensis* (classified as *N. curvifolia* by Schlimbach, 1924, cited in Snijman and Linder, 1996) and have since been observed in other species of *Nerine* (Snijman and Linder, 1996; see 5.3.1).

The large, fleshy, water-rich seeds are characteristic of members of the tribe Amaryllideae which, along with *Nerine*, includes *Amaryllis, Brunsvigia* and *Crinum* (Snijman and Linder, 1996). Another characteristic of this group is the absence of seed dormancy with vivipary reported in some species (Dahlgren *et al.*, 1985). The fleshy nature of the seed and absence of dormancy have important implications for plant breeders. In particular, seed storage and transport has been difficult. Additionally, as the seeds may be disseminated whilst the embryo is very small, factors that affect embryonic development, seed viability and germination need to be identified.

In addition to identifying optimal conditions for seed germination, tracking normal embryo development can also give plant breeders the basis on which to intervene when seeds from hybrid crosses break down prior to germination. This is a common occurrence in wide, inter-specific or inter-generic crosses (Ladizinsky, 1992). This breakdown is usually a result of chromosome imbalances in the embryo or endosperm (Monnier, 1995) and has been encountered in *Nerine* (Brown, unpublished). Understanding the ontogeny of the embryo provides a basis for comparison of growth with aborting embryos and allows identification of an appropriate stage for an intervention, such as embryo rescue. Embryo rescue has the capacity to save failing hybrid crosses by excising immature embryos from seeds, and aseptically culturing them *in vitro* on an artificial nutrient medium (Monnier, 1995).

In this chapter, the development of seed and embryo, from fertilisation through to the formation of a seedling, is described. Factors influencing germination such as light, substrate and temperature, have been investigated and implications are discussed.

5.2 Materials and methods

5.2.1 Seed development

All crosses were recorded and tagged to allow measurement of time to seed shed. Seed size was measured upon removal from the parent plant.

5.2.2 Embryo development

Embryo sections were obtained by hand-sectioning seeds or microtome-sectioning ovaries/seeds (see Appendix A.5.1). Hand-cut sections were stained with 0.01% Calcofluor (Calcofluor White M2R) and Acridine Orange (Solvent Orange, Sigma), dissolved in Walpole-HCl buffer (pH 3) (Clark, 1981; Appendix C.1). Microtomed material was fixed in 4% paraformaldehyde, dehydrated through an ethanol series and embedded in butyl methyl methacrylate (adapted from Webb and Gunning, 1990) or glycol methacrylate (Historesin ®, Jung) (Wikely and Goodsell, 1994; D. Wikely, pers. comm.). Small incisions were made into the seed coat to facilitate infiltration of the embedding solutions (M. Sedgley, pers. comm.). Sections were obtained using a Microm 340E rotary microtome, dried onto slides precoated with 3-Aminopropyltriethoxysilane (Sigma) and stained with Toluidine Blue or the DNA fluorochrome Hoechst 33258 (Sigma). The presence of starch was detected by staining with iodine solution. Sections were examined using a Zeiss Axiovert 35 inverted microscope fitted with an HBO mercury vapour lamp. An excitation filter BP365/12, dichroic beam splitter FT395 and barrier LP 397 were used for observation of fluorescence. Fluorescence micrographs were taken using Kodak Ektachrome Daylight 200 film; all other photographs were taken with Kodak Ektachrome 64T Tungsten film.

Early embryo size was measured by light microscopical observations of fluorescent stained material. After four weeks, seeds were dissected under a Zeiss Stemi SV stereo microscope into a graduated petri dish. Six to twelve seeds were dissected at weekly intervals, however, in early stages it was not possible to locate embryos in all seeds.

5.2.3 Seed germination

Germination data were obtained by weekly observation of seeds. Three media were used to examine effect of substrate: (i) seed raising mix supplemented with 25% coconut fibre; (ii) coconut fibre; and (iii) moist filter paper. Each substrate was tested at 20°C and 25°C. Germination trials were subsequently carried out on double sheets

of moist filter paper. Temperature trials used 30 seeds per temperature for *N. bowdenii* and 20 seeds per temperature for *N. sarniensis*. Viable seed was used in calculations.

5.2.4 Seed storage

Seeds were stored in clear plastic bags at 4°C, for periods ranging from one week to nine months. On removal from cold storage, seeds were placed on filter paper at 20°C, and germination observed for six months, or until all seeds had germinated.

5.2.5 Occurrence of parthenogenesis

Inflorescences of field grown plants of *N. bowdenii* x 'Clone 63', *N. flexuosa* 'alba' and *N. sarniensis* x 'Rosea', were emasculated prior to anthesis and bagged to prevent pollination. Inflorescences were subsequently examined for evidence of parthenogenesis.

5.2.6 Occurrence of albinism

All seedlings were examined at the one leaf stage to determine chlorophyll status.

5.3 Results

5.3.1 Seed development

In *Nerine*, development from pollination to seed maturation, marked by seeds bursting through the ovary wall, can take 3-9 weeks (Table 5.1; Plates 5.1 a-b). At maturity, the seeds are large, 3-8 mm in length (averages recorded in Table A.5.1), fleshy, globose to ovoid (Table A.5.2), with branched vascular tissue. The single integument consists of a chlorophyllous epidermal layer with stomata (Plate 5.1c), under which is chlorenchymous tissue grading to parenchymous tissue towards the centre of the seed (Plate 5.5). Starch is abundant and evenly distributed through the integument and endosperm.

Cultivar	Seed Shed	Germination
N. bowdenii x 'Clone 63'	4-8	4-11
N. flexuosa 'alba'	3-9	4-6
N. masonorum	3-4	3-5
N. sarniensis x 'Jill'	4-9	5-9
N. sarniensis x 'Rosea'	3-9	2-7

Table 5.1: Number of weeks from pollination to seed shed and seed shed to germination .

Numbers of seed produced per capsule is variable within and between species (see Appendix B: Breeding Records). In general, *N. sarniensis* cultivars present in the Channel Bulbs collection produce only a few (1-3) seeds per capsule, however, up to 25 seeds have been observed in a capsule of *N. sarniensis* x 'Pink Fairy'. Other *N. sarniensis* cultivars including x 'Indian Orange' (24) and x 'Gold Dust' (22) can also produce over 20 per capsule. *N. bowdenii* cultivars commonly produce 4-6 seeds per capsule, with up to 10 seeds being observed in *N. bowdenii* x 'Clone 63'.

The size of seeds is variable within and between cultivars of *Nerine* (Table A.5.1). *N. bowdenii* x 'Clone 63' produces seeds ranging from 3-8 mm in length, while seeds of *N. flexuosa* and *N. sarniensis* cultivars are smaller, ranging from 3-6mm. Seed size within a single cultivar varies, even when seeds have originated from the same ovary. However, not all seeds germinate and the likelihood of successful germination generally increases with seed size (small seed size may also be an indicator of parthenogenesis, see 5.3.5). In this study, seeds under 4mm in length (from all seed parents) had a markedly lower germination rate than seeds ≥4mm (Table 5.2). In *N. bowdenii* x 'Clone 63', *N. flexuosa* 'alba', as well as the *N. sarniensis* cultivars x 'Jill' and x 'Pink Opal', seeds ≥5mm had a significantly higher germination rate than 4-5mm seeds. *N. sarniensis* cultivars x 'Pink Fairy', x 'Rosea', x 'Sunset Falls' and x 'Xanthia' showed little or no difference in germination rates in seeds from the 4-5mm and ≥5mm classes. The *N. sarniensis* cultivars x 'Captain Dunne Cook', x 'Fred Danks' and x 'Brahms' produced smaller seeds with none measuring over 5mm. In the latter two, the 4-5mm seeds had germination rates of 93% and 100% respectively. [It should be noted that the sample size of x 'Brahms' was very small (2)].

SEED PARENT	% seed germination		
	2.5≤x<4mm	4≤x<5mm	≥5mm
N. bowdenii x 'Clone 63'	32 (28)	62 (29)	92 (49)
N. flexuosa 'alba'	0 (35)	28 (36)	60 (40)
N. sarniensis x 'Brahms'	0 (15)	100 (2)	-
N. sarniensis x 'Captain Dunne Cook'	25 (4)	50 (12)	-
N. sarniensis x 'Fred Dank'	14 (7)	93 (14)	-
N. sarniensis x 'Jill'	64 (11)	67 (15)	95 (42)
N. sarniensis x 'Pink Fairy'	8 (13)	47 (34)	53 (70)
N. sarniensis x 'Rosea'	19 (68)	68 (41)	85 (13)
N. sarniensis x 'Sunset Falls'	20 (5)	80 (24)	88 (17)
N. sarniensis x 'Xanthia'	75 (4)	100 (11)	100 (18)

Table 5.2: Average seed size and percentage seed germination for three size classes. Total number of seeds scored per size class in parentheses.

5.3.2 Embryo development

After fertilisation, approximately three days after pollination, the nuclear endosperm develops with an initial suspension of embryo growth (Plate 5.2a). Significant enlargement of the seed follows, predominantly due to cell division and enlargement in the multi-layer integument. At seed shed, a spherical proembryo measuring 100-150μm, with a clearly visible suspensor, is present at the micropylar end of the embryo sac (Plate 5.2b). The endosperm surrounds the embryo at this stage, however, it contributes a small proportion of the actual seed volume. The ontogeny is similar for all cultivars studied; starting with a proembryo and developing further after seed shed. However, the speed of development varies between species reflected by the differences in the timing of seed shed and subsequent germination (Table 5.1). The timing of developmental processes subsequently referred to is for *N. bowdenii* and is outlined in Table 5.5. Embryo size was measured at weekly intervals in *N. bowdenii* and is illustrated in Figure 5.1.

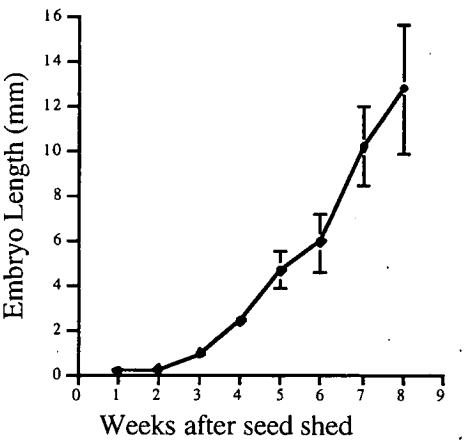


Figure 5.1: Mean length ± SE of *N. bowdenii* x 'Clone 63' embryo.

During the first two weeks after seed shed (W5[1]-W6), the developing embryo becomes globose and occupies the width of the embryo sac. The suspensor cells are still evident and the basal cell has undergone division (Plate 5.2c). At W6, one week after seed shed, the embryo is approximately 0.1mm in length and difficult to locate within the large seed. Polarity is evident at approximately W7, when the

[1] Time; W1-W17 refers to weeks after anthesis of the floret, and is outlined in Table 5.3.

embryo assumes an almond shape (Plate 5.2d). At these initial stages, there is little external or internal differentiation. By W8, embryo length is approximately 0.9mm; at this stage, an embryo can be excised with the aid of a dissecting microscope.

Differentiation becomes evident at W9, with the appearance of a distinctive epidermis in cross section (Plate 5.2e). The two poles of the embryo curl around forming a crescent shape, with a bulge developing in the centre (Plate 5.2e). The cells in this central bulge are much smaller than surrounding cells, suggesting they are rapidly dividing. During the next two weeks, a girdle of meristematic cells around the outside of the embryo becomes apparent. This coincides with the beginning of a period of rapid elongation of the embryo, which has now assumed a club shape.

Elongated procambial cells are apparent in longitudinal section at W10 (Plates 5.3a). Differentiation also occurs in the region that will emerge from the seed, with much of the meristematic activity occurring in the area behind the tip. Elongation and expansion continue until germination occurs in the following 2-3 weeks. The embryo remains small and uncoiled, occupying less than 10% of the seed volume at germination (Plate 5.1d).

At germination, which occurs in close proximity to the hilum, a tubular, non-chlorophyllous cotyledon emerges having a sheath-like, protective cap of cells at the tip (Plate 5.3b). In cross section, embryonic leaves are seen in the region below the tip (Plate 5.3c). The shoot apex is externally visible as a small notch, which is in close proximity to the emerging tip (Plate 5.3b). The cotyledon is positively geotropic, while the radicle pole undergoes swelling to form a probulb (Fig. 5.2). A contractile root emerges from the probulb and it develops into the major root during the first growth season (Fig. 5.2; Plate 5.4a). Its contractile properties act to pull the young probulb down into the substrate. The cotyledon becomes green after about one week. However, the tip remains embedded inside the seed (Fig. 5.2; Plate 5.23), which does not degenerate until well after the emergence of the first leaf, 3-5 weeks after germination. Generally, only 1-3 leaves form in the first season.

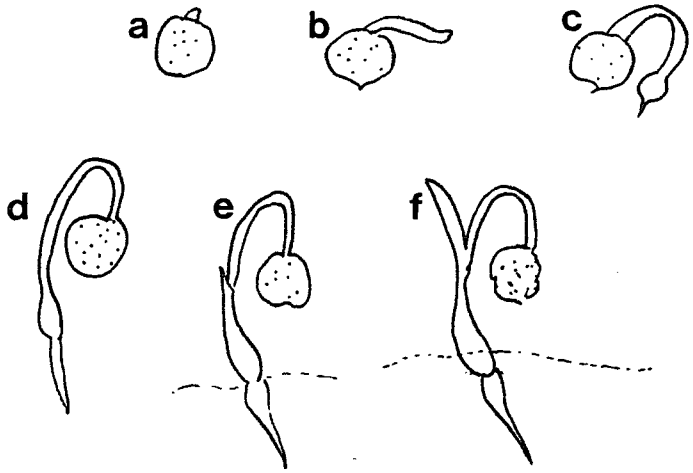


Figure 5.2: Germination of *Nerine* seed: a) cotyledon with protective cap emerges from the seed; b) tubular cotyledon elongates and becomes green in colour; c) probulb forms at distal end of the cotyledon; d) radicle develops into single broad contractile root; e) contractile root pulls probulb into the substrate; and f) leaf emerges.

There is substantial variation in the rate of embryo development in a given batch of seeds, although embryo size is relatively uniform for the first four weeks. By W11, seeds contained embryos ranging in size from an immature embryo of 2.5mm to a germinating embryo of 17mm. After germination, however, growth is extremely rapid accounting for the large size variation noted in Figure 5.1 after seven weeks. During this period seeds vary from ungerminated to those with emerging seedlings.

Weeks after anthesis	Event	Developmental stage
W0	Anthesis	
W1	Pollination	
W2		Fertilisation
W3		Enlargement of seeds
W4		
W5	Seed shed	Proembryo
W6		Globose embryo
W7		Polar embryo
W8		Epidermis evident
W9		External differentiation
W10		Rapid elongation/Procambium evident
W11		Embryonic leaves present
W12	Germination	
W13		External bulge at radicle pole
W14		
W15		Contractile root
W16		
W17		Leaf emergence

Table 5.3: Timeline of embryo development in *N. bowdenii* x 'Clone 63'.

5.3.3 Seed germination

Seeds can germinate at room temperature with no exogenous water or nutrient supply and in the absence of light. Seeds have been observed germinating on the laboratory bench and in seed envelopes kept on laboratory shelves (Plate 5.4b). In controlled conditions of 20°C and 25°C, no significant difference was found between germination percentage of seeds kept in light or dark conditions (Table 5.4). When testing three germination substrates (coconut fibre, filter paper and seed raising mix), no difference in the percentage germination was found at room temperature; germination percentages were above 95% in all cases (Table 5.5). However, some effect of substrate on germination was found at 25°C; 96% of seeds on the seed raising mix germinated, while the other two substrates had lower rates of 74% (filter paper) and 78% (coconut fibre) (Table 5.5).

Treatment	Germination viable seed (%)	
	20°C	25°C
Light	100 (15)	74 (27)
Dark	83 (29)	71 (17)

Table 5.4: Germination of *N. bowdenii* x 'Clone 63' in light and dark conditions. Substrate used was coconut fibre. Total number of seeds in parentheses.

Substrate	Germination of viable seed (%)	
	20°C	25°C
Coconut fibre	97 (38)	78 (41)
Filter paper	100 (37)	74 (46)
Seed raising mix	98 (41)	96 (46)

Table 5.5: Percentage germination of *N. bowdenii* x 'Clone 63' seed placed on three substrates at 20°C and 25°C. Total number of seeds in parentheses.

The time taken for germination was variable within and among the species studied, taking 2-11 weeks after seed shed (Table 5.1). *N. bowdenii* x 'Clone 63' was relatively slow to germinate and the timing of fresh seed germination varied considerably (4-11 weeks after seed shed). Faster germination occurred in *N. flexuosa* 'alba', *N. masonorum*, *N. sarniensis* x 'Jill' and *N. sarniensis* x 'Rosea'. Under identical conditions, germination time in *Nerine* can vary even when seeds are

collected on the same day (Fig. 5.3). Batches of seed from the fast germinating cultivar *N. sarniensis* x 'Rosea' exhibited 100% germination within a six week period, while it took nine weeks for 100% germination in *N. bowdenii* x 'Clone 63' (Fig. 5.3). A related species, *Amaryllis belladonna*, also possesses fleshy, water-rich seeds, which do not have a dormancy period. The chlorophyllous embryo is quite mature at seed shed and germination is rapid, occurring within three weeks of seed shed (Fig. 5.3).

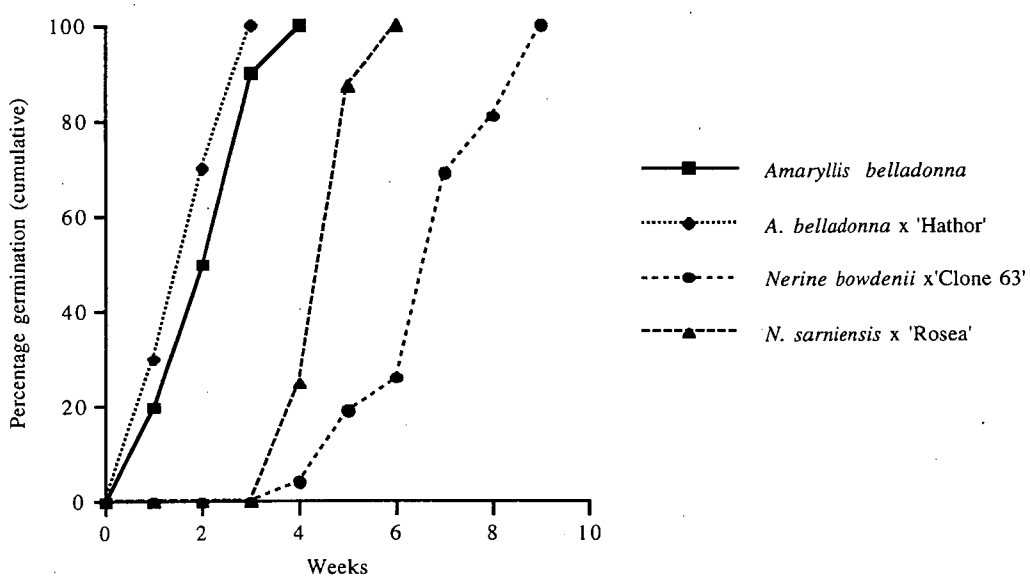


Figure 5.3: Germination of open pollinated seeds at room temperature.
(Time measured in weeks after seed shed.)

Seeds that degenerated within the first 3-4 weeks of the germination trial were assumed to be inviable. Embryos were not located in these seeds, supporting this assumption. Such seeds were commonly found in batches from *N. bowdenii* x 'Clone 63' and *N. flexuosa* 'alba', occurred rarely in *N. sarniensis* x 'Rosea' and *N. sarniensis* x 'Jill', and were not seen in *N. undulata.* The percentage of degenerated seed in any particular seed batch showed no correlation with treatment or seed age during the period of the study.

To determine if temperature affected seed germination, seeds of *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea' were incubated at temperatures ranging from 4-30°C (Table 5.6). Temperatures of 20-25°C were optimum for seed

germination in both cultivars tested, with germination rates of 100% well inside the trial period of three months. Interestingly, a two week exposure of 30°C was sufficient to cause seed degeneration in *N. bowdenii* x 'Clone 63'.

Temperatures of 10°C and 15°C did not affect actual percentage germination in either *N. bowdenii* x 'Clone 63' or *N. sarniensis* x 'Rosea', but did influence germination rate (Table 5.6). A temperature of 10°C caused the germination rate in both cultivars to decrease significantly. In *N. sarniensis* x 'Rosea', a period of four months was required for all seeds to germinate. This compares with less than two months at 20-25°C. A temperature of 15°C did not significantly slow germination in this cultivar. These low temperatures had a greater effect on full germination rate of *N. bowdenii* x 'Clone 63'; time taken for the germination of all seeds being seven months at 10°C and five months at 15°C (compared to 2-3 months at optimum temperature).

Effect on germination was more marked at 4°C. Seeds of *N. bowdenii* x 'Clone 63', *N. flexuosa* 'alba', *N. undulata* and *N. sarniensis* x 'Jill' can be stored at 4°C without germination. In *N. sarniensis* x 'Rosea' and *N. masonorum*, storage at 4°C will slow the germination rate, but some seed germination (40% over the three month trial in the case of *N. sarniensis* x 'Rosea') will occur (Table 5.6). This is also the case in *A. belladonna* where 4°C will slow, but not prevent, germination. In fact, all seeds of *A. belladonna* stored at 4°C germinated within a two month period (Table 5.7).

Cultivar	4°C	10°C	15°C	20°C	25°C	30°
N. bowdenii x 'Clone 63'	0 (0)	0 (100)	90 (100)	100 (100)	100 (100)	0 (0)
N. sarniensis x 'Rosea'	40 (100)	80 (100)	90 (100)	100 (100)	100 (100)	n/a

Table 5.6: Percentage germination after 3 months and after 7 months (in parentheses), at different temperatures for *N. bowdenii* x 'Clone 63'(winter dormant) and *N. sarniensis* x 'Rosea' (summer dormant).

5.3.4 Seed storage

Storage at 4°C for two months before transfer to 20°C did not affect percentage germination in *N. bowdenii* x 'Clone 63' and *N. sarniensis* x 'Rosea' (Table 5.7). The germination rate in both *N. sarniensis* x 'Jill' and *N. flexuosa* 'alba' showed a slight drop after two months of storage, yet remained over 70% (Table 5.7). *N. masonorum* did not show any drop in percentage germination after one month of

storage (Table A.5.4) Longer periods of storage, without significant effect on germination, were also possible in *N. bowdenii* x 'Clone 63', *N. sarniensis* x 'Jill', *N. sarniensis* x 'Rosea', *N. flexuosa* 'alba' and *N. undulata* (Tables 5.8, A.5.4 - A.5.7). In *N. flexuosa* 'alba' and *N. undulata* seed successfully germinated following removal from cold storage after six months. Effect of storage on *N. bowdenii* x 'Clone 63' was examined in more detail, with batches of seeds stored for up to nine months (Table 5.8). A high germination rate persisted, with percentage of viable seed germinating exceeding 80% for the duration of the study. Although the batches showed a variation in number of viable seed, there was no correlation between number of degenerating (inviable) seeds and length of storage (Table 5.9).

Periodical dissection of *N. bowdenii* seeds held at 4°C storage was performed to investigate embryo status. In all cases, embryo growth was suspended, which prevented sufficient development for germination. Even after 12 months storage, 39% of embryos were ≤0.5mm in length (Table 5.9). This is comparable to the percentage of this size class found after only four months storage and suggests embryo size does not increase after entering low temperature storage. Growth recommences when seeds are removed from cold storage and placed in 20°C, whereupon germination occurs as normal.

Species/Cultivar	No storage (control)	Cold storage
N. bowdenii x 'Clone 63'	100 (32)	100 (25)
N. flexuosa 'alba'	89 (35)	80 (20)
N. sarniensis x 'Jill'	95 (22)	75 (20)
N. sarniensis x 'Rosea'	100 (20)	100 (10)*
Amaryllis belladonna	100 (10)	100 (10)*

Table 5.7: Percentage germination of seeds cold stored for 2 months. Germination trial conducted for 12 months. * Seed germination occurred during cold storage. Number of seeds scored is in parentheses.

Storage Time	Germination % viable seed	Degenerated % total seed
No storage	100 (33)	6 (2)
1 week	95 (21)	16 (4)
2 weeks	100 (25)	0
3 weeks	86 (23)	23 (2)
4 weeks	100 (22)	27 (8)
2 months	100 (16)	36 (9)
3 months	100 (22)	13 (3)
4 months	96 (23)	7 (2)
5 months	83 (19)	5 (1)
6 months	95 (19)	5 (1)
7 months	89 (19)	5 (1)
8 months	85 (20)	0
9 months	93 (15)	25 (5)

Table 5.8: Germination of *N. bowdenii* x 'Clone 63' seed at 20°C after storage at 4°C. Germination trial conducted for 12 months. Number of seeds scored is in parentheses.

Storage period	Percentage of Embryos <0.5mm	0.5-1.0mm	>1mm
4 months	30	43	27
6 months	30	37	33
8 months	40	30	10
10 months	27	40	33
12 months	39	47	14

Table 5.9: Percentage of embryos in three size classes after storage at 4°C (30 seeds scored in each treatment).

5.3.5 Occurrence of parthenogenesis

Parthenogenetic seeds were regularly formed in bagged and emasculated inflorescences of *N. flexuosa* 'alba' (Table 5.10). The occurrence was much rarer in *N. bowdenii* x 'Clone 63', with less than 3% of bagged, emasculated inflorescences producing parthenogenetic seeds (Table 5.10). No instances were found in *N. sarniensis* x 'Rosea' (Table 5.10). The single known parthenogenetic seed produced by *N. bowdenii* x 'Clone 63' did germinate, but was albino (devoid of any chlorophyll - see 5.3.6). Of the 30 parthenogenetic seeds produced by *N. flexuosa* 'alba', all were less than 4mm and did not germinate *in vivo* on seed raising mix.

Cultivar	Parthenogenetic seeds	Control
N. bowdenii x 'Clone 63'	1 seed from 38 ovaries	66 seeds from 39 ovaries
N. flexuosa 'alba'	30 seeds from 50 ovaries	192 seeds from 124 ovaries
N. sarniensis x 'Rosea'	0 seeds from 53 ovaries	15 seeds from 160 ovaries

Table 5.10: Occurrence of parthenogenesis in bagged and emasculated inflorescences. Control inflorescences were left to open pollinate.

5.3.6 Occurrence of albinism

Albino seedlings have been found in cultivars of three species of *Nerine*; *N. bowdenii, N. flexuosa* and *N. sarniensis* (Table 5.11; Plate 5.4c). Seeds appear normal and upon germination the cotyledon emerges as normal (achlorophyllous). However, the cotyledon does not turn green as seen in normal seedling development. The basal end of the cotyledon remains embedded in the fleshy seed with seedlings able to continue development to the two leaf stage despite all tissues being devoid of chlorophyll. Leaf and root structures are regular in all respects apart from the lack of chlorophyll.

Of the crosses producing albino seedlings, seven out of ten also produced seeds with normal seedlings (Table 5.11). In all cases, these seedlings survived either *in vivo* (on seed raising mix) or *in vitro* (on tissue culture medium) (Table 5.11).

Cultivar	# albino	# in capsule	Others SRM	Germinated in TC
N. bowdenii x 'Clone 63' Open Pollinated	1	2	√	-
N. bowdenii x 'Clone 63' Parthenogenetic	1	1	-	-
N. flexuosa 'alba' **X** *N. filamentosa**	1	1	-	-
N. sarniensis x 'Mrs Cooper' **X** *N. sarniensis* x 'Pink Fairy'	1	1	-	√
N. sarniensis x 'Mrs Cooper' **X** *N. sarniensis* x 'Western Sunset'	1	3	X	√
N. sarniensis x 'Splendens' **X** *N. sarniensis* x 'Killi'	1	5	√	√
N. sarniensis x 'Jean O'Neill' **X** *N. sarniensis* x 'Galaxy'	1	13	√	-
N. sarniensis x 'Jean O'Neill' **X** *N. sarniensis* x 'Lady Lucy'	2	9	√	-
N. sarniensis x 'Brahms' Open Pollinated	1	9	√	√
N. sarniensis x 'Fred Dank' Open Pollinated	2	10	√	-

Table 5.11: Incidence of albinism in *Nerine* cultivars. * Possibly parthenogenetic seed. SRM = Germinated *in vivo*; TC = Germinated *in vitro*, X = Did not germinate, - = no results available.

5.4 Discussion

Nerine seeds are fleshy and do not undergo a dormancy period. Water-rich seeds are found in several members of the Amaryllidaceae including *Clivia*, *Haemanthus*, *Hymenocallis*, *Amaryllis*, *Boophane* and *Brunsvigia*. The seeds of the latter three genera are bitegmic, unlike the unitegmic seeds of the tribe Amaryllideae (Meerow and Snijman, 1998), including *Nerine* (Dahlgren *et al.*, 1985). *Crinum* seeds, also fleshy, differ anatomically from *Nerine*, in that the outer layers of the endosperm become corky during development (Howell and Prakash, 1990). In *Nerine*, the integument increases after fertilisation, in part due to cell division. This also occurs in *Hymenocallis* (Whitehead and Brown, 1940), but in the latter genus it is integumentary tissue, rather than endosperm that constitutes most of the seed volume. The genus *Amaryllis* possesses a similar fleshy, non-dormant seed to *Nerine*, however, embryo development is quite different. At seed shed, the *Amaryllis* embryo is chlorophyllous and mature with recognisable organs, contrasting dramatically with the immaturity of the *Nerine* embryo. Consequently, it is not surprising that germination occurs much more quickly in *Amaryllis*.

The *Nerine* zygote undergoes a resting period following fertilisation during which time the nuclear endosperm develops. The resting period of zygotes with nuclear endosperm is generally longer in duration than that of species with cellular endosperm (Bhatnar and Johri, 1972). In the case of *Nerine*, this resting period appears to be lengthy with significant increase in the integument also occurring during this period, immediately after fertilisation. At seed shed, the embryo of *Nerine* is at the proembryo stage and development proceeds in a period of after-ripening that may take 3-10 weeks. Development of the embryo progresses from a radially symmetrical proembryo, through a globose stage and a bipolar almond shape, before assuming a club shape at maturity. The axial symmetry of the proembryo is common in monocotyledons being maintained until the differentiation of the shoot apex (Sourges, 1935, cited in Bhatnar and Johri, 1972). The embryo is linear (Martin, 1946, cited in Bhatnar and Johri, 1972), a common characteristic of the Amaryllidaceae (Dahlgren and Clifford, 1982). The widest pole of the mature embryo remains embedded in the seed, while the opposite pole elongates, and emerges from the seed.

At germination, the embryo is rudimentary in external morphology, achlorophyllous and has no leaf or root structures. The achlorophyllous nature of the embryo of *N.*

bowdenii, contradicts the findings of Snijman and Linder (1996) who found green embryos in *Nerine* species. A chlorophyllous embryo was thought by these authors to be consistent in all members of the Amaryllideae. However, as a complete survey of *Nerine* species has not been undertaken, it is possible that both chlorophyllous and achlorophyllous embryos are present in the genus. The lack of differentiation of the embryo into organs is unusual; normally mature embryos have a recognisable radicle, plumule and cotyledon (Bhatnagar and Johri, 1972). Nevertheless, reduced embryos (without the usual organisation) have been observed in other species including *Ranunculus ficaria* (Sourges, 1913; cited in Maheshwari, 1950).

Nerine seeds do not degenerate until true leaves have formed, suggesting that seeds may be important as nutrient sources for the small seedling. This is supported by the fact that germination and seedling development can occur in the absence of moisture, light or exogenous nutrients. This pattern of germination is similar to that reported in *Hymenocallis* (Whitehead and Brown, 1940) and *Crinum* (Isaac and McGillivray, 1965). The presence of a stomatose epidermis and sub-epidermal chlorenchymous tissue suggests that the integument may be a carbohydrate source for the developing embryo/seedling. Starch is thought to be the most important nutrient compound in water-rich seeds and is commonly found in both endosperm and integument (Dahlgren *et al.*, 1985). In *Hymenocallis*, starch is manufactured by the chlorenchymous tissue of the integument (Whitehead and Brown, 1940). The origin of starch in the integument remains to be determined in *Nerine*, but it may also originate from the chlorenchymous integumentary tissue.

The action of the contractile tap root to pull the probulb into the substrate, also seen in *Crinum* (Pate and Dixon, 1982), ensures that the bulb will remain covered by soil in future growth phases. This may be a survival adaptation to pull the bulb below the surface for protection prior to the dormant phase. The positioning of the *Nerine* probulb at depth is interesting in light of numerous reports suggesting that the genus has a horticultural requirement for bulbs to be planted, with neck and a substantial part of the bulb, out of the ground (*e.g.* Harrison and Harrison, 1967; Yates, 1994). Importantly, this does not appear to occur in the natural environment. Nevertheless, although not a requirement for growth, planting bulbs out of the ground in commercial operations has implications for disease control, in particular to avoid overwatering and consequent risk of fungal infections as well as allowing for spraying with pesticide (I. Warrington, pers. comm.).

Nerine seeds with their water and carbohydrate reserves, appear to be well adapted to adverse conditions. This is evident by the fact that seeds can germinate and reach the two leaf stage in the absence of light, water and nutrient substrate. Temperature, however, does appear to be critical to germination with the optimum temperature for germination is approximately 20°C. Temperatures of 10°C and 15°C retard germination, but do not adversely affect seed viability. *N. sarniensis* x 'Rosea' is less affected by cool temperature than the slower germinating *N. bowdenii* x 'Clone 63' and will germinate even in a cool 4°C, although germination rate is slower. All species were found to be sensitive to high temperature with seeds becoming overgrown with fungal infection and degenerating at 30°C. Seeds germinated at 25°C, but the germination percentage was considerably reduced, when using filter paper or coconut fibre as a substrate, in comparison with that at 20°C,. Use of seed raising mix as a substrate at this temperature did not result in a decrease of germination rate, which may have been due to the insulating and porous nature of the substrate allowing for moisture retention. The increased insulatory and water retention properties of coconut fibre over filter paper may also explain why seeds had a slightly higher germination rate on coconut fibre at this temperature. These results suggest that *Nerine* seeds are sensitive to desiccation.

Seeds characterised by a high water content and non-acquisition of desiccation tolerance are classed as recalcitrant (Neves, 1994; Kermode, 1997) and typically lose viability once moisture content drops below a critical value (Neves, 1994; Bannister *et al.*, 1996). Species with recalcitrant seeds include the commercially important *Camellia sinensis* (tea), *Theobroma cacao* (cocoa) and *Arrocarpus hererophyllus* (jackfruit) (Chandel *et al.*, 1995) as well as species of the genus *Quercus* (Berjak *et al.*, 1999; Sun, 1999; Tommasi *et al.*, 1999). The inability of recalcitrant seeds to tolerate desiccation has implications for storage both conventionally and using cryopreservation (Berjak *et al.*, 1999; Sun, 1999). A review of storage methods for a number of species possessing recalcitrant seeds has been completed by Neves (1994) with work continuing on individual species to determine critical moisture levels and storage temperatures for successful preservation (*e.g.* Chandel *et al.*, 1995; Normah *et al.*, 1997). It has been found in some species that particular stages of physiological maturity are less susceptible to desiccation, and may be successfully stored, similarly, embryonic axes of some species may be successfully excised and cryopreserved (*e.g.* Chandel *et al.*, 1995).

Several characteristics found in *Nerine* seeds have been reported in other species with recalcitrant seeds. Non-dormancy and a high germination rate is also found in the tropical fruit species *Garcinia mangostana*, *Baccaurea motleyana* and *B. polyneura* (Normah *et al.*, 1997), a slowing of metabolic activity of seeds in low temperatures has been found in *Aisandra butyracea* (Dhar *et al.*, 1999) and excessive fungal growth and rotting at high temperatures shown to occur in *Combretum bracteosum* (Dalling and van Staden, 1999).

N. bowdenii x 'Clone 63', *N. undulata* and *N. flexuosa* 'alba' seeds can be stored at 4°C without germinating. This is due to the suspension of embryo growth. Importantly, seeds retain their viability and are able to continue development after removal from storage. *Amaryllis belladonna*, however, can germinate at 4°C, with only a slowing of germination. This phenomenon may be due to the well developed embryo having passed a commitment point where development cannot be suspended, so growth and the germination process continue albeit at a slower rate than in optimum conditions. A similar situation may also exist in *N. sarniensis* x 'Rosea'. As this is a fast germinating cultivar, the after-ripening period is much shorter, and it is possible embryos have developed to a critical stage, prior to arriving in cool storage. Consequently, the germination process continues, although slowed down by the cooler temperature. *N. masonorum* also germinates rapidly; this may also explain why this species will germinate in cool conditions. Although a likely scenario, the above is difficult to confirm, as identification of embryo stage requires destruction of the seed.

According to Whitehead and Brown (1940), the after-ripening condition of seeds is intermediate between true vivipary and seeds with dormancy mechanisms (usually being dehydrated and requiring water to break dormancy). The water-rich seeds of *Nerine* are usually shed from the parent plant and require further development (after-ripening) prior to germination. This condition is also true of *Hymenocallis* and *Crinum*, but is different from true viviparous seeds, where germination occurs whilst remaining connected to the parent plant. However, there are reports of seeds of *Nerine* (Dyer, 1976; Sherriff, 1994), *Crinum* (Howell and Prakash, 1982) and *Hymenocallis* (Newton, 1985) germinating before being shed. Howell and Prakash (1982) suggest that these seeds, with occasional precocious germination, may be an evolutionary precursor to vivipary.

The necessary period of after-ripening, and ability to suspend embryo growth in low temperature, may be an adaptation to environmental conditions. As *Nerine* is an autumn-flowering genus, seed set occurs in late autumn/early winter. The suspension or retardation of embryo growth in cold conditions could help seedlings survive adverse winter conditions in the absence of a seed dormancy mechanism. The fact that the slower developing species *N. bowdenii* is found in montane districts, where winter temperatures are very low, lends support to this idea. Delaying germination until temperatures are rising may also be an advantage where surface soil is frozen. In such conditions, the contractile tap root would be able to penetrate the surface without damage and pull the probulb under the substrate.

In *N. bowdenii*, seeds can be cold stored at 4°C, and seed germination will occur once the seeds are moved to warmer temperatures. In the first three months of storage at 4°C, all seeds successfully germinated, as did those planted immediately after harvest. This suggests that these seeds could survive a cold winter and subsequent embryo growth/seed germination could occur in warmer spring temperatures. Seeds of other *Nerine* species/cultivars also could be cold-stored; *N. flexuosa* 'alba' and *N. undulata* showed no decrease in percentage germination after six months in cold storage. Similarly, these seeds may also be adapted to survive adverse winter conditions. Unfortunately, *N. sarniensis* x 'Jill' seeds were not as amenable to cold storage as other varieties, with some seeds shrivelling during cold storage. Although germination rate was still quite high (75%), it was markedly lower than that found using fresh seeds. This may be a result of the seeds not being adapted to survive periods of suspended animation due to cold. The natural habitat of *N. sarniensis* would not experience the same harsh winters endured by *N. bowdenii*, so seeds may not be capable of suspending growth for long periods. This may also explain why growth cessation does not occur in *N. sarniensis* x 'Rosea' at low temperatures. As both these varieties are hybrids, an alternative possibility is that these observations are due to adaptive properties of the seed being lost in the hybridisation process.

The ability to store seeds, without adversely affecting germination, is of extreme horticultural significance. This viability lasts for at least nine months in *N. bowdenii* (Table 5.8), and for at least six months in *N. undulata* (Table A.5.7). This would allow use of cool temperatures to slow/suspend germination for later planting or for long-distance seed transportation.

There is considerable variation in seed production rates within the genus. *N. bowdenii* produces 1-10 seeds per floret, all of which are round with a diameter exceeding 4mm, whilst *N. sarniensis* x 'Indian Orange', x 'Salmon Supreme' and x 'Sunset Falls' can produce over 20 seeds per capsule, which are much smaller in volume. The germination rates of these seed batches are both high, so it appears neither of the two strategies offer a survival advantage over the other. It may be that size is related to length of time required for the completion of embryo development, and therefore is related to time taken for germination. At least in the case of *N. bowdenii* x 'Clone 63', the seeds are slower to germinate than the smaller-seeded *N. sarniensis* x 'Rosea'.

The small seed yields (1-3) observed in many cultivars, particularly those of *N. sarniensis*, may stem from lack of pollinators or reduced pollen flow (see Chapter 4), or some degree of fertility may have been lost due to prolonged vegetative propagation of the line. Certainly, the production of large numbers of seeds per floret do not affect the chances of survival of a single seed, providing the seeds are greater than 4mm in length.

The concept of a minimum size requirement for successful germination of *Nerine* seed is of significance to plant breeders. Seeds not achieving viable size may be due to: (i) ovules developing without being fertilised (parthenogenesis); (ii) seeds not having developed sufficiently due to late pollination; (iii) arrested development due to lack of maternal resources through competition with other seeds or florets; or (iv) breakdown of the embryo or nutrient endosperm as a result of a hybrid cross. In the latter three cases, seeds would have been fertilised and their development may be assisted by artificially supplying nutrients to allow continuation of development. This can be achieved by the culture of small seeds on a nutrient tissue culture medium. Seeds less than 4mm have developed to germination stages on tissue culture media (Brown *et al.*, 1998). Consequently, this technique is extremely promising especially for seeds from hybrid crosses. Preliminary experiments have indicated that germination rates of small seeds can be improved by tissue culture (see Chapter 7). However, one major obstacle is that it is impossible to determine categorically whether an ovule has actually been fertilised.

Embryo rescue is another promising technique available to plant breeders who wish to save failing crosses. However, the ontogeny of the *Nerine* embryo has significant implications for this technique. Successful embryo rescue usually requires embryos

at the post-globular stage with survival of *in vitro* embryos generally increasing with maturity. However, aborting embryos must be excised prior to a breakdown of the endosperm. Therefore, a balance between these two factors must be achieved (Monnier, 1995). In *Nerine*, the post-globular stage can be several weeks after seed shed (*e.g. N. bowdenii*), by which time the endosperm could have already significantly broken down. Isolation and culture of the immature embryo at the time of seed shed requires a specialised culture medium or combination of media for development into a seedling (Brown *et al.*, 1998). Successful embryo rescue has been achieved in embryos less than 1mm dissected from *N. bowdenii* x 'Clone 63', although current success rates are relatively low (Brown *et al.*, 1998). It is likely that the use of ovule culture may be an appropriate alternative for use in *Nerine*, at least until the embryo has reached an appropriate size for embryo rescue. Embryo rescue has been successful in *Hippeastrum* (Bell, 1973) and *Amaryllis belladonna* (see 6.3.3), however, the embryo in these genera is significantly more developed than the *Nerine* embryo at the stage of isolation.

The use of tissue culture techniques, such as ovule culture and embryo rescue, may also assist in determining the mechanism responsible for the development of parthenogenetic and albino seeds. The supply of artificial nutrients in culture may support the growth of embryos from these seeds which otherwise would not survive. This would then permit analysis for ploidy level or genetic markers.

The appearance of seeds in which chlorophyll is absent has not previously been reported in *Nerine*. Designation of these white seedlings as 'albino' is in accord with the *albina* mutation described in *Pisum* (Blixt, 1972). In *Nerine*, normal development of albino (achlorophyllous) seedlings proceeds normally to the 2-leaf stage, presumably due to a supply of nutrients available within the fleshy seed. It may therefore be possible to support the continued growth of albino seedlings by transfer to tissue culture, from which analysis of ploidy level or genotype would be possible. Unfortunately, such transfer has thus far not been successful due to problems encountered in decontamination of the seedlings. Disinfestation of seedlings established in soil is difficult due to the presence of soil-borne pathogens, and requires a disinfestation regime that severely damages the tissues of a small seedling. This problem may be overcome by germinating seed batches that may contain albino seedlings (from crosses outlined in Table 5.11), in tissue culture, where seeds are disinfested prior to germination. This can be successfully achieved (see Chapter 7).

Alternatively, seeds may be placed on clean filter paper for germination and screening. Unfortunately, this could not be completed as flowering problems in the subsequent years of this study did not permit these crosses to be performed.

The cause of albinism in *Nerine* may be due to the expression of a recessive gene. Occurrence of albinism in self crosses, is consistent with the notion of a recessive gene. Ratios of albino seedlings in multi-progeny crosses also do not contradict this possibility. It is also interesting to note that the cultivar *N. sarniensis* x 'Mrs Cooper' formed these seeds from two different crosses. If albinism is due to the expression of a recessive gene, it should occur more commonly with certain parent plants.

A second possibility is that albinism is a form of late acting incompatibility or hybrid breakdown. This appears to occur following inter-specific hybridisation in other genera including *Zantedeschia,* where albinism has been encountered in hybrids which have survived due to embryo rescue (Yao *et al.*, 1995). Interestingly, albino and chlorophyll deficient seedlings have also been found in hybrid seedlings of *Trifolium* (Pandey, 1957; Pandey *et al.*, 1987; Przywara *et al.*, 1989) and these have generally appeared in only one cross direction, with the reciprocal cross being normal (Przywara *et al.*, 1989). In *Nerine*, reciprocal crosses were only possible in two of the ten crosses producing albino seedlings. In both of these normal seedlings were produced from reciprocal crosses. This is consistent with the findings in *Trifolium*, and warrants further investigation to determine if this is a widespread phenomenon in *Nerine*. Production of albino seedlings after a hybrid cross, thought to be caused by incompatibility between the plastid genome and the genome (Kirk and Tilney-Basset, 1978, cited in Yao *et al.*, 1995), is a powerful isolating mechanism that will eliminate all progeny (Przywara *et al.*, 1989). However, if all hybrid progeny do not present this phenotype, the efficiency of this mechanism for genetic isolation is significantly reduced. This is the case in *Nerine*, where seeds from crosses producing albinos have also produced seeds that develop normally.

Whether albinism is related to parthenogenesis remains to be determined. Certainly, in the only known parthenogenetic seed to germinate in this study (*N. bowdenii* x 'Clone 63') was achlorophyllous (albino). In addition, of the albino seedlings listed in Table 5.11, several seeds may have been parthenogenetic in origin. For example, the cross *N. flexuosa* 'alba' **X** *N. filamentosa* is most probably parthenogenetic, as *N. filamentosa* pollen has not generated any other successful crosses, and *N. flexuosa*

'alba' has been found to regularly produce parthenogenetic seeds. Parthenogenesis cannot be ruled out even when other ovules from the same cross have been fertilised: it is not possible to distinguish between parthenogenetic and fertilised seeds in this species. A relationship between haploidy and albinism has been found in gynogenic and androgenic plantlets of *Oryza sativa* and *Tritcum aestivum*. In these species both albino and green plants have been regenerated (Yang and Zhou, 1982).

Parthenogenesis has been previously reported in *Nerine* by Hannibal (1959) in both *N. humilis,* where pollen is required to trigger seed production (*i.e.* induced apomixis [Johansen, 1950]), and *N. bowdenii*, where pollination is not required for seed production (*i.e.* autonomous apomixis [Johansen, 1950]). Other genera in the Amaryllidaceae (*Habranthus*, *Cooperia*, *Zephyranthes* and *Sprekelia*) exhibit some form of apomixis, presumably parthenogenesis (Flory, 1939; Traub, 1974; Sharma and Thorpe, 1995). Results from this study indicate that autonomous apomixis operates in *N. flexuosa* 'alba' and *N. bowdenii,* as pollen is not required to trigger seed production. The precise pathway of parthenogenesis is yet to be determined. In *N. bowdenii* x 'Clone 63', parthenogenetic seeds will germinate, however, those of *N. flexuosa* 'alba' have not done so under the conditions of this study. Whether this parthenogenetic seed will germinate under different conditions (*e.g. in vitro*) remains to be determined.

The occurrence of parthenogenesis, has both advantages and disadvantages for plant breeders. The controlled production of parthenogenetic seeds can assist a breeding programme by producing homozygous individuals. However, parthenogenesis may also occur when a controlled pollination has been made with the desired outcome being a hybrid. Unfortunately, it is not possible to morphologically distinguish between a parthenogenetic seed and a fertilised seed, when harvesting controlled crosses or open pollinations. This is also a problem in other parthenogenetic genera such as *Lilium* (North and Wills, 1969). In cultivars such as *N. flexuosa* 'alba' and *N. bowdenii* x 'Clone 63', where parthenogenesis occurs quite commonly, it is therefore difficult to be sure whether any seedlings produced are genuine hybrids. This has probably been the case in the past when purported hybrids have closely resembled the seed parent. It may also explain incidences where seeds have been formed, but germination did not occur.

5.5 Conclusion

The seeds of *Nerine* are fleshy and have no dormancy period. Following successful fertilisation, a nuclear endosperm develops and the integumentary tissue increases. At seed shed, a proembryo is present and the majority of embryo development occurs during a period of after-ripening of 2-11 weeks duration. At germination, the embryo is rudimentary, with a non-chlorophyllous cotyledon emerging from the seed and the shoot apex visible as a small notch. The positively geotropic cotyledon forms a pro-bulb from which a contractile root develops. Emergence of the first true leaf occurs 3-5 weeks after germination.

Immaturity of the *Nerine* embryo at the time of seed shed may be of adaptive significance, in that growth can be suspended or retarded during periods of low temperature. This property can be utilised by horticulturalists who wish to store seeds without affecting viability. However, it can prove disadvantageous to hybridisers wishing to employ embryo rescue to save failing crosses. Nevertheless, whole ovule culture appears to be a possible solution in these situations.

Germination data reported in this study has significance to growers, in particular regarding optimum germination conditions. As exposure of seeds to greater than 20°C can cause degeneration, it would be beneficial to use an insulating substrate to facilitate germination in regions where temperature may fluctuate above this level.

Albino seedlings have been found in cultivars of three *Nerine* species. Development is normal to the two-leaf stage whereupon these seedlings perish. Whether albinism is related to parthenogenesis, which has also been identified in *Nerine*, remains to be determined.

Plate 5.1a Swollen ovaries of *Nerine* with developing seeds. Scale bar = 1cm.

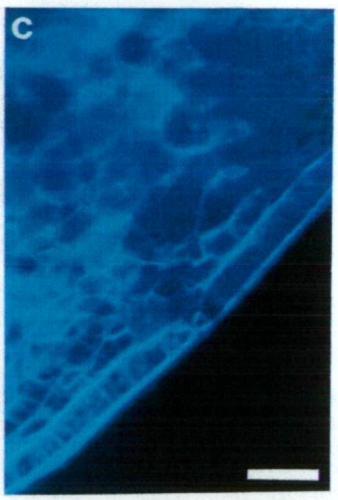
Plate 5.1b Seeds bursting through the ovary/fruit walls of *N. masonorum.* Note asynchronous nature of floret/fruit development. Scale bar = 4cm.

Plate 5.1c Cross section of *Nerine* seed with guard cells and associated stoma in epidermis. Scale bar = 100μm. (Stain: Calcofluor.)

Plate 5.1d Cross section of *N. bowdenii* x 'Clone 63' seed showing embryo (e), one week prior to germination. Integumentary tissue consists of chlorenchymous tissue (c) and inner parenchymatous tissue with vascular elements (v). Hilum (h) is also visible. Scale bar = 2cm.


a


b


c

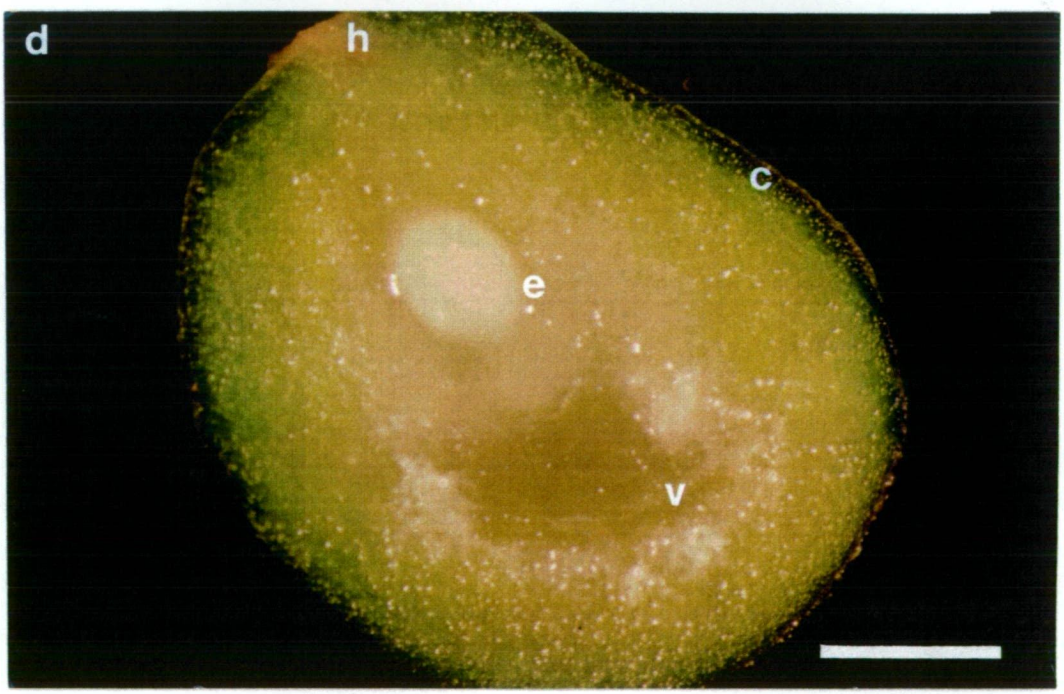

d
h
c
e
v

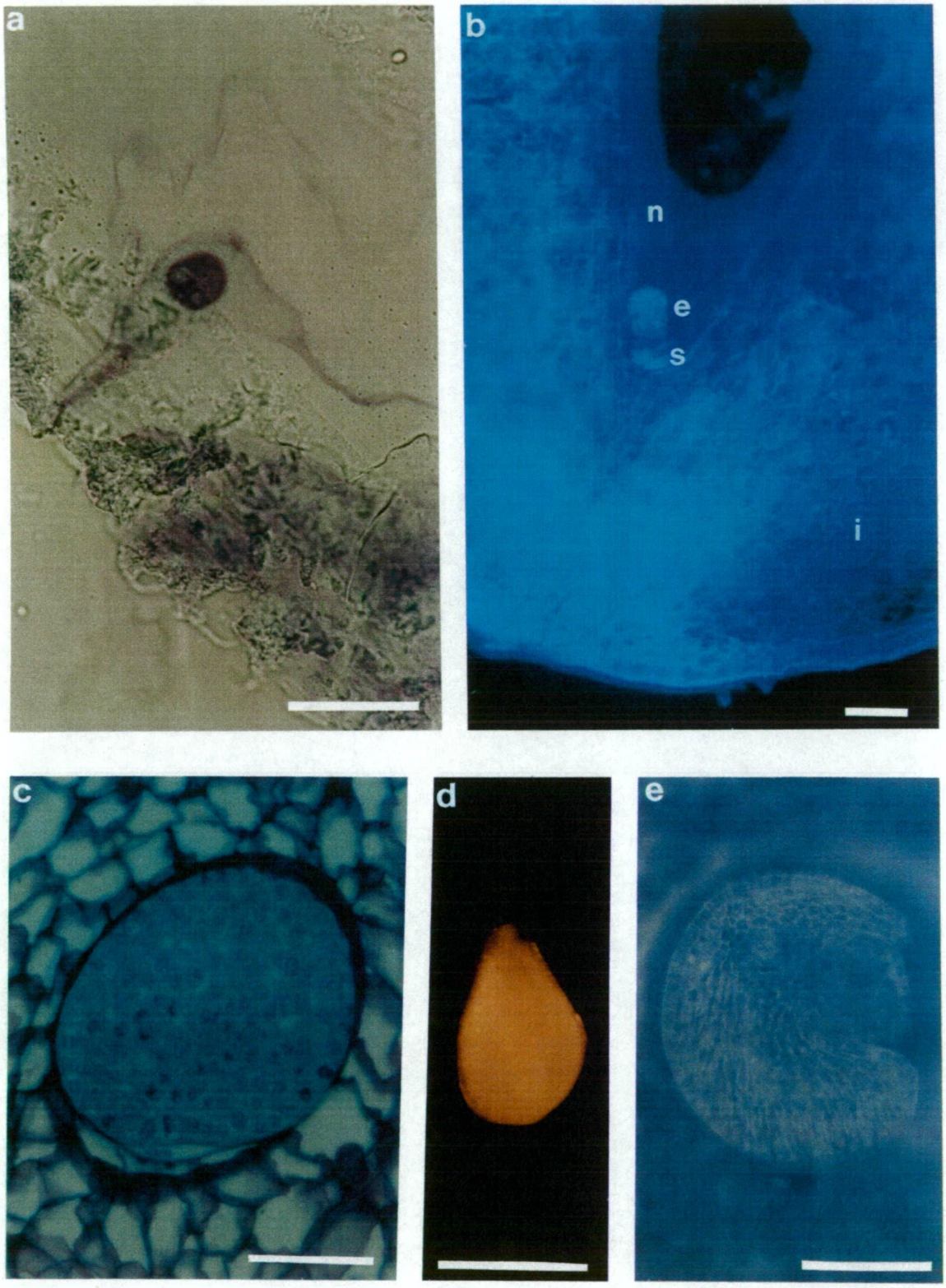
Plate 5.2a *N. sarniensis* x 'Rosea' zygote. Scale bar = 100μm. (Stain: Toluidine blue.)

Plate 5.2b Longitudinal section of *N. masonorum* seed showing pro-embryo (e) and suspensor cells (s) at seed shed. Also evident are the multilayer integument (i) and the endosperm (n). Scale bar = 100μm. (Stain: Calcofluor.)

Plate 5.2c Longitudinal section of *N. masonorum* seed one week after seed shed. Embryo is globular and has expanded to fill the embryo sac. Suspensor cells can be seen at the micropylar end. Scale bar = 100μm. (Stain: Toluidine blue.)

Plate 5.2d Almond shape embryo, excised from the seed. Scale bar = 500μm. (Unstained.)

Plate 5.2e Longitudinal section of *N. bowdenii* x 'Clone 63' embryo six weeks after seed shed. The embryo is polar, curling inwards to form a crescent shape, with central bulge of dividing cells. A distinct epidermis is visible. Scale bar = 100μm. (Stain: Calcofluor.)


a
b
n
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s
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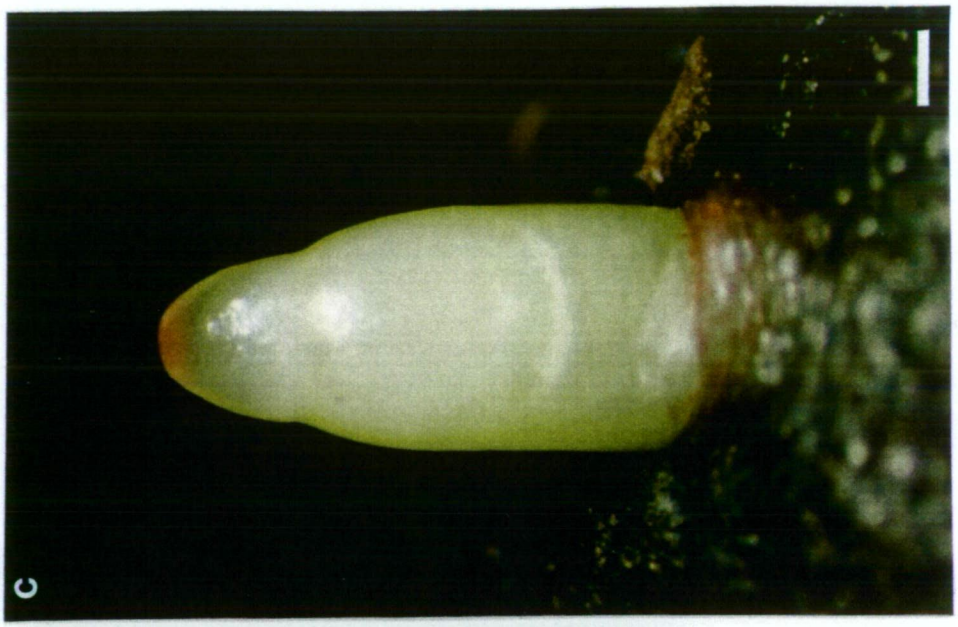
e

Plate 5.3a Longitudinal section of *N. bowdenii* x 'Clone 63' embryo, dissected from the seed six weeks after seed shed. Note the elongated procambial cells (p). Scale bar = 500μm. (Stain: Toluidine Blue.)

Plate 5.3b Longitudinal section of *N. bowdenii* x 'Clone 63' embryo at germination. The cap of protective cells is evident at the tip. Embryonic leaves are seen at the shoot apex positioned behind the tip. Scale bar = 100μm. (Stain: Calcofluor.)

Plate 5.3c Tip of embryo emerging from seed, two days after germination. Scale bar = 500μm.


c


b

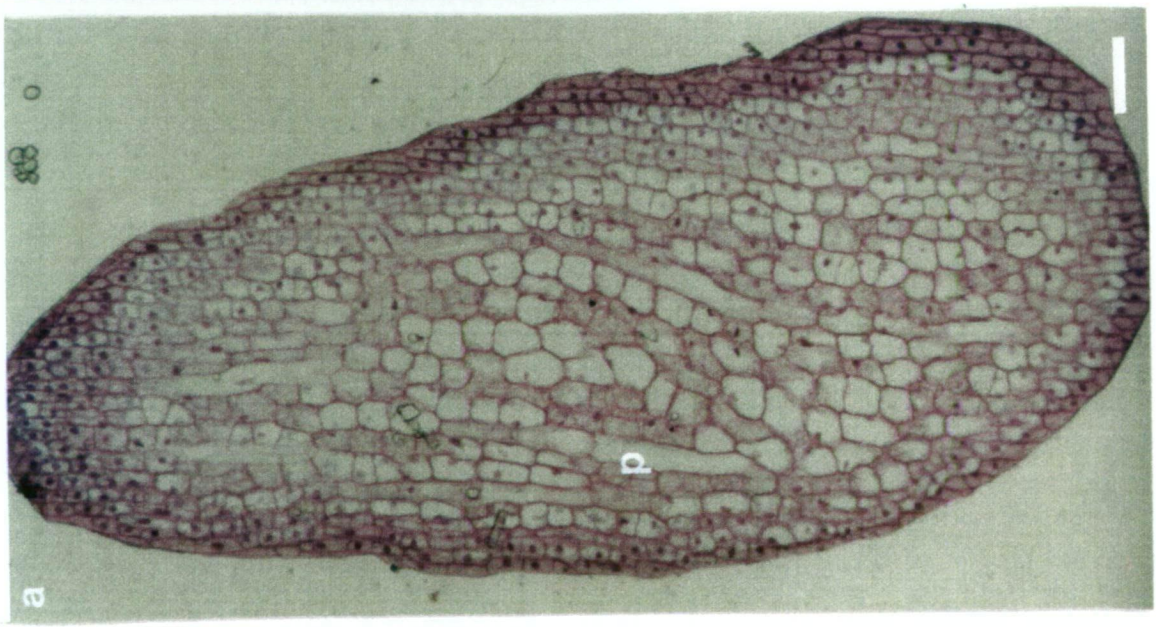

o
p
a

Plate 5.4a Germinated seedling of *N. bowdenii* x 'Clone 63' three weeks after germination. The well-developed contractile root is emerging from the swelling probulb. One true leaf has emerged and the seed has yet to degenerate. Scale bar = 0.5cm.

Plate 5.4b Seedlings of *N. bowdenii* x 'Clone 63' germinating on the laboratory shelf. Note the proliferation of root hairs and the premature disintegration of the seed. Scale bar = 2mm.

Plate 5.4c Albino and chlorophyllous seedlings of *Nerine* in tissue culture. Scale bar = 1cm.


b
c
a

Appendix A.5.1: A note on sectioning methods used for *Nerine* seeds.

The large, fleshy seeds of *Nerine* present problems when attempting to observe early embryo development. In particular, it is difficult to achieve good infiltration of the tissue when using resin embedding. An alternative method for microscopic observation, using thicker hand-cut sections, and staining with fluorochromes was developed to locate the embryo. The use of thicker sections prevents the problem of the embryo 'popping out' of the embryo sac, and being lost. Use of fluorochromes in preference to conventional staining allows the use of thicker sections by permitting observation of the cut surface only.

The above technique can be quickly and easily utilised to screen for embryo size and developmental stage. Once embryo position is found, it is possible to use resin embedding with a smaller sub-section of the seed, which allows for greater infiltration of the resin.

A modified technique for resin embedding has also been developed. The seed coat was nicked in several places with a scalpel blade, immediately prior to the fixation stage (M. Sedgley, pers. comm.). All stages of fixation, dehydration and infiltration were carried out under vacuum. Once the specimen was embedded in the resin, the mould was sealed with hot paraffin wax.

Cultivar	Size (mm)	Cultivar	Size (mm)	Cultivar	Size (mm)
N. bowdenii x 'Clone 63'	5.6±0.1 (121)	*N. sarniensis* x 'Early Snow'	4.3±0.2 (14)	*N. sarniensis* x 'Mrs Cooper'	4.7±0.9 (15)
N. flexuosa 'alba'	4.8±0.2 (45)	*N. sarniensis* x 'Enchantress'	4.5±0.2 (17)	*N. sarniensis* x 'Novelty'	5.0±0.2 (12)
N. undulata x 'Roseo-crispa'	3.6±0.2 (4)	*N. sarniensis* x 'Eve'	4.8±0.2 (10)	*N. sarniensis* x 'Pink Fairy'	4.8±0.1 (137)
N. masonorum	4.3±0.1 (40)	*N. sarniensis* x 'Fred Danks'	3.9±0.1 (23)	*N. sarniensis* x 'Pink Jewel'	3.15±0.1 (11)
N. sarniensis x 'Angela Limerick'	3.8±0.3 (15)	*N. sarniensis* x 'Fothergillii major'	4.7±0.3 (9)	*N. sarniensis* x 'Pink Opal'	3.9±0.4 (7)
N. sarniensis x 'Brahms'	3.6±0.2 (7)	*N. sarniensis* x 'Gold Dust'	4.1±0.1 (28)	*N. sarniensis* x 'Redhead'	4.0±0.2 (12)
N. sarniensis x 'Cpt. Dunne Cook'	3.8±0.1 (15)	*N. sarniensis* x 'Imp'	5.1±0.1 (14)	*N. sarniensis* x 'Rosea'	4.2±0.1 (88)
N. sarniensis x 'Caroline'	4.1±0.2 (9)	*N. sarniensis* x 'Indian Orange'	3.6±0.1 (52)	*N. sarniensis* x 'Salmon Supreme'	4.45±0.1 (52)
N. sarniensis x 'Chorister'	5.3±0.1 (31)	*N. sarniensis* x 'Jean O'Neill'	3.9±0.1 (44)	*N. sarniensis* x 'Splendens'	3.7±0.5 (3)
N. sarniensis x 'Corusca'	5.2±0.1 (22)	*N. sarniensis* x 'Jill'	4.2±0.1 (29)	*N. sarniensis* x 'Sunset Falls'	4.1±0.1 (75)
N. sarniensis x 'Cuckfield'	3.75±0.2 (8)	*N. sarniensis* x 'Kenilworth'	4.4±0.2 (22)	*N. sarniensis* x 'Western Sunset'	3.6±0.2 (6)
N. sarniensis x 'Cynthia Chance'	5.6±0.5 (5)	*N. sarniensis* x 'Killi'	4.4±0.1 (36)	*N. sarniensis* x 'Xanthia'	4.6±0.2 (33)
N. sarniensis x 'Donna'	5.8±0.5 (4)	*N. sarniensis* x 'Mother of Pearl'	5.5±0.1 (2)		

Table A.5.1: Average seed size for species and cultivars of *Nerine*. Number of seeds measured in parentheses.

Round		Oval		Kidney Shaped
Green	Brown/red + green	Green	Brown/red + green	Green
N. flexuosa	*N. bowdenii* x 'Clone 63'	*N. sarniensis* x 'Jill'	*N. sarniensis* x 'Rosea'	*N. sarniensis* x 'Brahms'
N. undulata x 'Roseo-crispa'	*N. bowdenii* x 'Pink Jewel'	*N. undulata*	*N. sarniensis* x 'Angela Limerick'	*N. sarniensis* x 'Chorister'
	N. masonorum		*N. sarniensis* x 'Corusca'	*N. sarniensis* x 'Enchantress'
				N. sarniensis x 'Fothergillii major'
				N. sarniensis x 'Pink Fairy'
				N. sarniensis x 'Salmon Supreme'
				N. sarniensis x 'Sunset Falls'

Table A.5.2: Categories of seed shape and colour.

Storage time (months)	Germination % (#)	Disintegrated %	Germination viable seed %
0	90 (10)	0	90
1	90 (10)	0	90

Table A.5.3: Seed viability after storage 4°C for *N. masonorum.*

Storage time (months)	Germination % (#)	Disintegrated %	Germination viable seed %
0	100 (40)	0	100
1	85 (20)	5	89
2	70 (20)	5	73
3	70 (20)	25	95

Table A.5.4: Seed viability after storage 4°C for *N. sarniensis* x 'Jill'.

Storage time (months)	Germination % (#)	Disintegrated %	Germination viable seed %
0	100 (20)	0	100
2	100 (10)	0	100
3	100 (5)	0	100

Table A.5.5: Seed viability after storage 4°C for *N. sarniensis* x 'Rosea'.

Storage time (months)	Germination % (#)	Disintegrated %	Germination viable seed %
0	78 (37)	5	85
1	85 (20)	10	94
2	70 (20)	25	95
3	40 (10)	40	67
4	50 (10)	20	63

Table A.5.6: Seed viability after storage 4°C for *N. flexuosa* 'alba'.

Storage time (months)	Germination % (#)	Disintegrated %	Germination viable seed %
0	100 (6)	0	100
1	100 (3)	0	100
6	100 (3)	0	100

Table A.5.7: Seed viability after storage 4°C for *N. undulata.*

Chapter Six

Hybridisation in *Nerine.*

6.1 Introduction

Hybridisation is the natural or artificial fusion of two genetically different gametes during fertilisation leading to gene flow between populations which are reproductively isolated to various extents (Rieger and Michaelis, 1958, cited in Hermsen, 1992; Ladizinsky, 1992). This reproductive isolation is achieved by a variety of physical, mechanical and physiological mechanisms which have evolved to preserve the species as a breeding group (Clarke and Knox, 1978).

Hybridisation can operate on several levels, but in practice it is usually confined to inter-familial, inter-generic, inter-specific or intra-specific interactions. Inter-familial crosses are rarely successful due to differences in genome homology preventing the development of the zygote. Although possible, inter-generic crosses are generally prevented, while inter-specific are often successful, at least in the first generation (Hermsen, 1992). Intra-specific crosses generally succeed except where specific recognition genes (self-incompatibility genes) operate to prevent inbreeding (Heslop-Harrison, 1975; Clarke and Knox, 1978).

The use of inter-specific and inter-generic crosses is a valuable method of introducing new genetic variation into cultivated plants, and can subsequently lead to improved varieties for commercial markets. For flower bulbs it is inter-specific hybridisation which is the most important source of new genetic variation (van Tuyl, 1997). Inter-specific crosses have resulted in many new cultivars of cut flowers in genera such as *Lilium*, *Narcissus* and *Tulipa* (van Eijk *et al.*, 1991; van Creij *et al.*, 1992).

The horticultural potential of *Nerine* has long been recognised, with inter-specific hybridisation programs beginning in the mid to late nineteenth century (see Table A.6.1). The first named inter-specific hybrids, raised by Dean Herbert, were published in Baker's 1888 monograph *Handbook of the Amaryllideæ* (Table A.6.1). The variation present in the genus and in the Amaryllidaceae family (consisting of 17 genera [Russell *et al.*, 1985, cited in Courtze and Louw, 1990]) continues to be recognised by breeders as a source for improved varieties (Tables A.6.2, A.6.3). Consequently, programs to breed for specific horticultural traits are currently being continued worldwide (Coertze and Louw, 1990; van Brenk and Benschop, 1993).

In the past, inter-generic crosses using *Nerine* have, in the main, been attempted by horticulturalists and collectors, largely for novelty. Several hybrids have been successful, particularly those using the closely related members of the sub-tribe Amaryllidinae (Tables A.6.4, A.6.5). The first known inter-generic hybrid using *Nerine* was by Bidwell in the early 1900s who successfully crossed *N. bowdenii* with *Amaryllis belladonna* (Gallagher, 1967). Several inter-generic crosses using the Amaryllidaceae are now on the market, most notably Amnerine (*A. belladonna* **X** *N. bowdenii*) and Ammarcrinum (*A. belladonna* **X***Crinum sp.*) (Doutt, 1994). Nevertheless, barriers to crossing do occur at the inter-generic level, so certain crosses have not been achieved using conventional means. Use of *in vitro* technology (see Chapter 7) has opened up greater possibilities for wide hybrids, and efforts towards this end are continuing (Coertze and Louw, 1990; Toussaint, 1995).

Inter-specific crosses are generally easier to achieve than inter-generic crosses due to the species being more closely related. Certainly, a number have been previously achieved in *Nerine* (Table A.6.6). The majority of the inter-specific crosses achieved to date use cultivars from Section Bowdeniae and Section Nerine (see Table 1.1.2; Fig. 6.1). This is probably due to both accessibility and horticultural suitability. Alternatively, physical or physiological barriers may prevent the use of some *Nerine* species and account for the small number of species which have featured in inter-specific and inter-generic crosses to date. In other genera, crossing barriers do regularly occur at this level (*e.g. Brassica* [Inomata, 1982]; *Phalaris*, *Trifolium* and *Ornithopus* [Williams *et al.*, 1982]; *Medicago* [McCoy and Smith, 1988]; *Tulipa* [van Creij *et al.*, 1992]) so it would not be unexpected when crossing more distantly related species of *Nerine*.

Achievement of a wide cross is often only the first step in a breeding programme, with wide hybrids in themselves not generally useful for horticulture. Their main use is for ongoing breeding work, particularly backcrossing in order to breed for desirable characteristics. Therefore, ongoing crosses are often important to breeding programmes. To date, hybridisation in *Nerine* has rarely gone beyond first generation, at both intra- and inter-generic level. This is largely due to fertility problems encountered in hybrids, preventing crossing from proceeding past the first or second generation.

Fertility problems encountered in hybrids are often due to chromosome imbalances from the unrelated parents. This may involve progeny no longer having a diploid chromosome number. During the cultivation and breeding of *Nerine* [2n = 22], a number of polyploids have arisen, including *N. sarniensis* x 'Fothergillii major' (triploid) and *Nerine* x 'Inchmery Kate' (tetraploid). These have become integral in controlled breeding programmes as many early hybrids were the result of crosses between triploids and diploids. Consequently, a number of *Nerine* hybrids are aneuploid or polyploid in either the first generation (*e.g.* x 'Excellence' [2n = 28], a cross between *N. flexuosa* [2n = 22] and *N. humilis* 'major' [2n = 33] [Ammal, 1951]) or second generation (*e.g.* x 'Exonia' [2n = 22], a cross between *N. sarniensis* x 'Fothergillii' [2n = 33] and *N. bowdenii* [2n = 22], used in further hybridisation giving rise to x 'Aurora' [2n = 33] and x 'Hera' [2n = 33] [Ammal, 1951]). The triploid cultivars (x 'Aurora', x 'Hera', *N. sarniensis* x 'Fothergillii major' [Plates 6.1a]) are amongst the most beautiful nerines, therefore their use in future hybridisation is desirable. Unfortunately, their ploidy level may be a barrier to normal fertility.

Chromosome imbalances, and consequent low fertility, is also possible when the resultant hybrid is diploid. This is particularly true of wider crosses, such as those at inter-generic level (*e.g.* x 'Fletcheri' [2n = 22], a cross between *Amaryllis belladonna* and *Nerine bowdenii* [Ammal, 1951]). This cultivar has a pollen sterility rate as high as 96% (Ammal, 1951). Chromosome imbalances in hybrids may therefore account for many *Nerine* cultivars which set very little, if any, seed or which have pollen of low fertility. A survey of *Nerine* in cultivation by Roberts (1984) has determined the presence of 68% diploid, 15% aneuploid, 15% triploid and 2% tetraploid cultivars. Presently, the frequency of different ploidy levels in wild populations is unknown. However, the possibility that an extended period in

cultivation, where vegetative propagation precludes the necessary selection of fertility, cannot be discounted. Whatever the cause, infertility or reduced fertility affects both the seed and pollen parents in *Nerine*. Therefore, establishment of fertility prior to choice of both seed and pollen parents in crosses is critical.

In fertile parent plants, important reproductive biology data such as timing of receptivity and pollen longevity needs to be determined (see Chapter 3). Once established it is necessary to perform controlled crosses to identify the outcome of the cross. It is difficult to prove unequivocally that two parents are uncrossable as they may be crossable under certain conditions, or by the use of certain genotypes (Hermsen, 1992). Nevertheless, if point of failure can be identified in a cross, a hybrid may be possible with some manipulation. Stebbins (1958) has identified two levels at which crosses can break down: pre-zygotic and post-zygotic (for review of reproductive barriers see Shivanna, 1982; Williams *et al.*, 1987; Khush and Brar, 1992).

In natural populations, pre-zygotic barriers include isolation mechanisms such as spatial isolation and temporal displacement of flowering time. However, in controlled crossing programmes, it is the physical and physiological barriers occurring in the pistil to prevent fertilisation, which are of primary concern. Incompatibility systems are a common physiological pre-zygotic barrier which can act at both intra-specific and inter-specific level (Lewis, 1947; Brewbaker, 1957; Crowe, 1963; Heslop-Harrison, 1975; Trognitz and Schmiediche, 1993). Recognition of incompatible pollen may occur at stigma, style or ovule level and result in arrest of germination or pollen tube growth, or retardation of pollen tube growth so fertilisation is unable to be affected prior to floral senescence (*e.g.* van der Valk *et al.*, 1991). A physical constraint such as style length can also prevent pollen tubes from effecting fertilisation (Khush and Brar, 1992).

Whilst physical barriers can often be easily overcome (see Chapter 7), the presence of incompatibility systems can cause difficulties for hybridisers, in particular for inter-specific breeding. The term incompatibility is generally confined to those systems which recognise and reject self pollen preventing too close a union. However, there is evidence to suggest the same locus may prevent too remote a union, when inter-specific crosses are attempted (Trognitz and Schmiediche, 1993). In the latter cases, pattern of inhibition of pollen tube growth resembles that of gametophytic self-incompatibility (Ladizinsky, 1992). This particular form of recognition occurs mainly

in crosses of self-incompatible (SI) species with self-compatible (SC) species, and usually occurs unidirectionally with the reciprocal cross being successful (Dhaliwal, 1992). In *Nerine*, crosses successful in only one direction have been reported (Smee, 1984), although whether it is related to unilateral incompatibility is yet to be determined.

Post-zygotic barriers occur after fertilisation and include breakdown of the embryo or endosperm, breakdown of the hybrid prior to reaching reproductive maturity or infertility of the hybrid (*e.g.* Williams and de Latour, 1980; Khush and Brar, 1992; Yao *et al.*, 1995; van Tuyl *et al.*, 1997). This is usually due to chromosome imbalances in the hybrid genome.

Identification of point of failure is essential in order to devise an appropriate technique of intervention to overcome the barrier. Microscopical techniques, such as fluorescent staining of pollen tubes in the stigma, style, and ovary, as well as sectioning of ovules assist in identifying where the cross is breaking down prior to fertilisation. Evidence for post-zygotic barriers includes production of seeds which do not germinate, aborted embryos in seeds and hybrids that breakdown prior to flowering or are unable to produce fertile gametes.

Amassing information on fertility, receptivity and results of hybrid crosses can provide a scientific base on which to build a hybridisation program. By incorporating interventionist techniques, hybrids not possible through conventional breeding may be generated. Importantly, these techniques may allow the program to extend beyond first generation hybrids.

6.2 Materials and methods

6.2.1 Parent plants used in this study

Intra-specific crosses

Cultivars of *N. sarniensis* and *N. bowdenii* (see Appendix B: Breeding Records).

Inter-specific crosses

Cultivars of *N. sarniensis* and *N. bowdenii*, *N. undulata* (see Appendix B: Breeding Records), *N. filamentosa, N. flexuosa* 'alba' and *N. masonorum* (Plates 6.1 b-d).

Inter-generic crosses

Cultivars of *N. sarniensis* and *N. bowdenii* (see Appendix B: Breeding Records), *Amaryllis belladonna*, *A. belladonna* 'Hathor', *Brunsvigia josephinae*, *Cyrtanthus elatus*, *Lycoris aurea* and *L. radiata* (Plates 6.2 a-e).

6.2.2 Assessment of fertility of hybrid cultivars

6.2.2.1 Malformation of reproductive organs

The inflorescences of all cultivars in the collection were examined for the presence of ovules and normal pollen-producing anthers.

6.2.2.2 Pollen fertility

In vitro germination of pollen

Pollen was cultured *in vitro* and examined for evidence of germination (see 4.2.3.2).

Light microscopical examination of pollen grains

Fresh pollen grains, from six cultivars, were examined by light microscopy to identify evidence of sterility. Pollen from at least two anthers was examined, with more than one plant used where possible. Five randomly selected fields of view were scored.

Controlled crossing

All cultivars in the Channel Bulbs collection were used, where possible, as pollen parents in controlled crossing (see Appendix B).

6.2.2.3 Ovule fertility

All cultivars in the Channel Bulbs collection were used, where possible, as seed parents in controlled crossing, including self-pollination (see Appendix B). Following flowering, all cultivars were examined for any evidence of open pollination.

6.2.3 Determination of self-compatibility

Controlled crosses, using the procedure described in 4.2.4.1, were carried out using self pollen. Crosses were repeated, where possible, and stigmata of varying age used where available (Appendix B).

6.2.4 Controlled crossing to determine success of hybridisation

Procedure was as described in 4.2.4.1. Crosses were performed at intra-specific, inter-specific and inter-generic level. Where possible reciprocal crosses were also undertaken. When material permitted, crosses were repeated and stigmata of more than one age used. When the period of stigmatic receptivity was unknown, crosses were carried out between A and A+4. A full list of crosses is contained in Appendix B.

Seedlings were raised in 50:50 coconut fibre/seed raising mix at room temperature

6.2.5 Identification of point of failure in hybrid crosses

6.2.5.1 Pre-zygotic barriers

Pollen tube growth

Styles were excised one week after pollination, fixed in EAA (3 parts 70% ethanol: 1 part glacial acetic acid) for four hours and stored in 70% ethanol. Preparation for microscopy was as described in 4.2.3.1.

Reciprocal crosses

Where possible, reciprocal crosses were performed.

Style measurements

Measurements of style length were carried out prior to pollination, at the stage when the first whorl of anthers were open.

6.2.5.2 Post-zygotic barriers

Non-germinated seed

Seed germination was recorded (see Appendix B) to identify crosses in which a seedling was not produced.

6.3 Results

6.3.1 Assessment of fertility of hybrid cultivars

6.3.1.1 Malformation of reproductive organs

Sterility may manifest in obvious deformity of the reproductive structures, such as empty anthers (Plate 6.3a), sparse pollen production and/or absent or malformed ovules (Table 6.1). In many cultivars, reproductive organs appeared to be normal, yet no seed was set from either open pollinations or controlled crosses. In these cultivars, sterility cannot be categorically concluded, however, they are almost certainly of extremely low fertility.

Ovules absent or malformed	Anthers empty of malformed	Pollen sparse	No seed set from any cross or by open pollination
x 'Guy Fawkes'	x 'Elvira'	x 'Aurora'	*N. filamentosa*
x 'Lucinda'	x 'Gladys Dettman'	x 'Hera'	*Cyrtanthus elatus*
x 'Mansellii'		x 'Old Rose'	*Lycoris aurea*
			Lycoris radiata

Table 6.1: Physical manifestation of infertility of *Nerine* cultivars and related Amaryllids in the Channel Bulbs collection.

6.3.1.2 Pollen fertility

Pollen fertility was assessed via controlled crossing for 60 cultivars held in the Channel Bulbs collection (Table A.6.7). These crosses have indicated pollen of some *Nerine* cultivars appears to be inviable. This has been supported by *in vitro* germination trials for 30 cultivars, which have found viability ranging from: 0% (*e.g.* x 'Ancilla', x 'Aurora', x 'Salmonea'); below 20% (*e.g.* x 'Old Rose', x 'Curiosity'); 20-60% (*e.g.* x 'Fothergillii major', x 'Optimist'); or above 60% (*e.g.* x 'Corusca', x 'Latecomer', x 'Pink Jewel').

Light microscopical examination of pollen grains

Instances of low fertility or sterility can occur when the inflorescence appears structurally normal and pollen is present. However, light microscopical observation of pollen has revealed that grains can lack cell contents (Plate 6.18; Table 6.3). The pollen grains of six cultivars of *Nerine* were examined with all showing empty pollen grains. However, the fertile cultivars such as *N. flexuosa* 'alba' and *N. sarniensis* x 'Rosea' had a very low percentage of empty grains (1% and 2% respectively) (Table 6.2). The apparently infertile cultivars *Nerine* x 'Ancilla' and x 'Cameo Beauty' had very high rates of empty grains (100% and 90% respectively) (Tables 6.1; 6.2). Examination of pollen grains from two closely related genera, *Amaryllis* and *Brunsvigia* found no empty pollen grains in the randomly selected samples (Table 6.2).

Species/Cultivar	% empty pollen grains
Nerine x 'Ancilla'	100
Nerine x 'Cameo Beauty'	90
Nerine x 'Fothergillii major'	62
Nerine x 'Lucinda'	50
Nerine x 'Pink Jewel'	2
Nerine x 'Rosea'	2
N. flexuosa 'alba'	1
Amaryllis belladonna	0
Brunsvigia josephinae	0

Table 6.2: Percentage of empty pollen grains found in *Nerine* cultivars and related species.

6.3.1.3 Ovule fertility

Ovule fertility was assessed in cultivars via controlled crossing, observations of open pollinations and examination of ovule morphology (Table 6.1). A summary of fertility, for each species and cultivar in the collection as determined by these observations, is presented in Table A.6.8. A number of cultivars did not set seed in any controlled crosses or as a result of open pollinations (*e.g.* the inter-generic cross *Nerine* x 'Fletcherii [Plate 6.3b]) (Appendix B: Breeding Records). Others set seed infrequently (*e.g. N. sarniensis* x 'Fothergillii major'). In those which did not set seed, there was evidence of reduced or malformed ovules (*e.g. Nerine* x 'Lucinda' [Table 6.1]).

6.3.2 Self-compatibility

Self-compatibility was determined in 24 *Nerine* cultivars (Table 6.3).

Self-compatible cultivars	
N. bowdenii x 'Clone 63'	*N. sarniensis* x 'Jean O'Neill'
N. bowdenii x 'Winter Cheer'	*N. sarniensis* x 'Jill'
N. flexuosa 'alba'	*N. sarniensis* x 'Kenilworth'
N. sarniensis x 'Angela Limerick'	*N. sarniensis* x 'Mrs Bromley'
N. sarniensis x 'Brahms'	*N. sarniensis* x 'Pink Fairy'
N. sarniensis x 'Cuckfield'	*N. sarniensis* x 'Pink Opal'
N. sarniensis x 'Cynthia Chance'	*N. sarniensis* x 'Rosamund Elwes'
N. sarniensis x 'Eve'	*N. sarniensis* x 'Rosea'
N. sarniensis x 'Fothergilli major'	*N. sarniensis* x 'Sunset Falls'
N. sarniensis x 'Fred Danks'	*N. sarniensis* x 'White Dove'
N. sarniensis x 'Gold Dust'	*N. sarniensis* x 'Xanthia'
N. sarniensis x 'Indian Orange'	*N. undulata* x 'Roseo-crispa'

Table 6.3: Self-compatible (SC) cultivars in Channel Bulbs *Nerine* collection.

Non-flowering of many cultivars in the final two years of this study meant, in many cases, number of self crosses were too low to conclusively determine the existence of SI. This also meant follow-up microscopical observations of possibly SI cultivars was impossible. Consequently, the number of SC cultivars is most probably under-estimated. In addition, in cultivars where self crosses have been unsuccessful, it may be to infertility rather than SI.

Controlled crosses of *Nerine* species and other amaryllids held in the Channel Bulbs collection has established the presence of SC in seven species (Table 6.4).

Taxon	Outcome
*N. bowdenii**	Self-compatible
N. flexuosa 'alba'	Self-compatible
N. filamentosa	No selfs successful**
N. masonorum	Self-compatible
*N. sarniensis**	Self-compatible
N. undulata	Self-compatible
Amaryllis belladonna	Self-compatible
Brunsvigia josephinae	Self-compatible
Cyrtanthus elatus	No selfs successful**
Lycoris aurea	No selfs successful**
L. radiata	No selfs successful**

Table 6.4: Occurrence of self-compatibility in the Channel Bulbs Collection. * Majority of fertile cultivars which have been tested are SC
** No seed produced from any cross.

In *N. filamentosa*, *Cyrtanthus elatus*, *Lycoris aurea* and *L. radiata,* no self pollinations were successful, but no other seeds were produced by open pollination or controlled crossing in a number of individual bulbs (*Lycoris aurea* [30], *Lycoris radiata* [5], *Cyrtanthus elatus* [200] and *N. filamentosa* [40]). This sheds some doubt on the fertility of the bulbs held in the collection. As such, SI cannot be concluded.

6.3.3 Controlled crossing to determine success of hybridisation

Records of all crosses performed in this study are outlined in Appendix B.

6.3.3.1 Intra-specific crosses

Ninety-six successful (*i.e.* seedling producing) intra-specific crosses between *N. sarniensis* cultivars are listed in Table A.6.9. In addition, seven others were successful when fertilised ovules were introduced into tissue culture (see Chapter 7). In some cases seeds did not go on to germinate (see 6.3.4). The complete record of all intra-specific crosses performed during this study is contained in Appendix B.

6.3.3.2 Inter-specific crosses

Eleven inter-specific crosses successful produced seedlings via traditional crossing methods (Table 6.5), while one other was successful when the fertilised ovule was introduced into tissue culture (see Chapter 7). In addition, several crosses produced seed which did not germinate (see 6.3.4). Crossing relationships are summarised in a crossing polygon (Fig. 6.1). The complete record of all inter-specific crosses performed during this study is contained in Appendix B.

Inter-specific Cross
N. bowdenii x 'Clone 63' **X** *N. sarniensis* x 'Jean O'Neill'
N. bowdenii x 'Clone 63' **X** *N. sarniensis* x 'Jill'
N. bowdenii x 'Clone 63' **X** *N. sarniensis* x 'Killi'
N. bowdenii x 'Clone 63' **X** *N. sarniensis* x 'Pink Opal'
N. bowdenii x 'Clone 63' **X** *N. sarniensis* x 'Rosea'
N. bowdenii x 'Pink Jewel' **X** *N. flexuosa* 'alba' *
N. bowdenii x 'Pink Jewel' **X** *N. sarniensis* x 'Rosea'
N. flexuosa 'alba' **X** *N. bowdenii* x 'Clone 63'
N. flexuosa x 'alba' **X** *N. sarniensis* x 'Mrs Cooper'
N. flexuosa x 'alba' **X** *N. undulata*
N. masonorum **X** *N. bowdenii* x 'Pink Jewel'
N. sarniensis x 'Rosea' **X** *N. bowdenii* x 'Clone 63'

Table 6.5: Successful (seedling producing) inter-specific crosses (first named is seed parent). * Seedling only germinated in tissue culture (see Chapter 7).

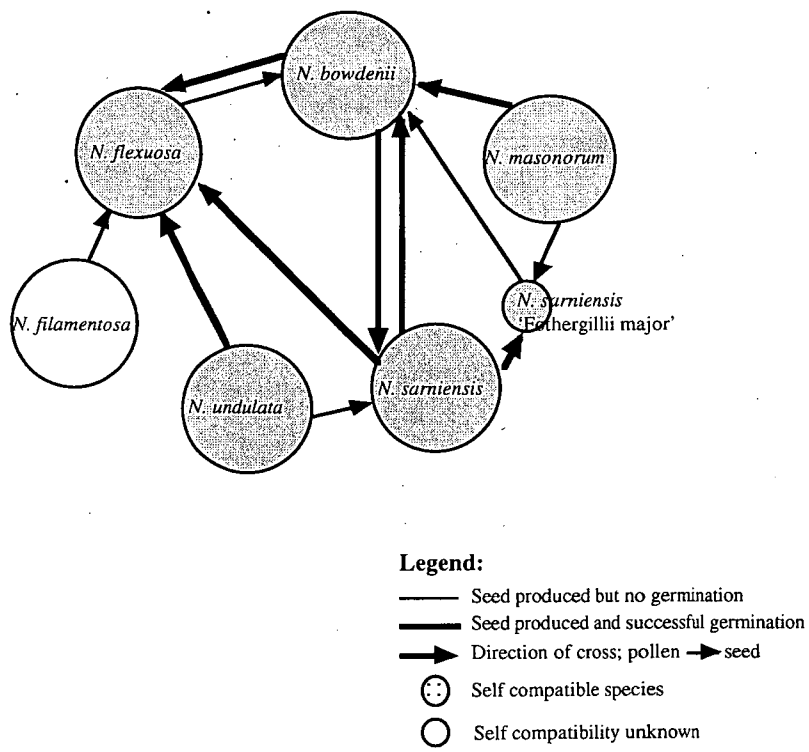


Figure 6.1: Crossing polygon for *Nerine* species in Channel Bulbs collection.

6.3.3.3 Inter-generic crosses

Inter-generic crosses were attempted between three species of the Tribe Amaryllideae (*Amaryllis belladonna*, *Brunsvigia josephinae* and *Nerine spp.*), and the more distantly related genera *Cyrtanthus* and *Lycoris* (see Appendix B). The eight seed producing crosses are outlined in Table 6.6, and crossing relationships are summarised in a crossing polygon (Fig. 6.2).

Cross	Result
Amaryllis belladonna **X** *N. bowdenii* x 'Pink Jewel'	Seedling produced
Brunsvigia josephinae **X** *Amaryllis belladonna* x 'Hathor'	Seedling produced
Brunsvigia josephinae **X** *Cyrtanthus elatus*	Seedling produced
Nerine sarniensis x 'Rosea' **X** *Lycoris aurea*	Seed - no germination
Nerine sarniensis x 'Rosea' **X** *Amaryllis belladonna*	Seedling produced
Nerine sarniensis x 'Rosea' **X** *Brunsvigia josephinae*	Seed - no germination
Nerine sarniensis x 'Cynthia Chance' **X** *Amaryllis belladonna*	Seedling produced
Nerine sarniensis x 'Fothergillii major' **X** *Amaryllis belladonna*	Seed - no germination

Table 6.6: Results of inter-generic crosses where seed was produced.

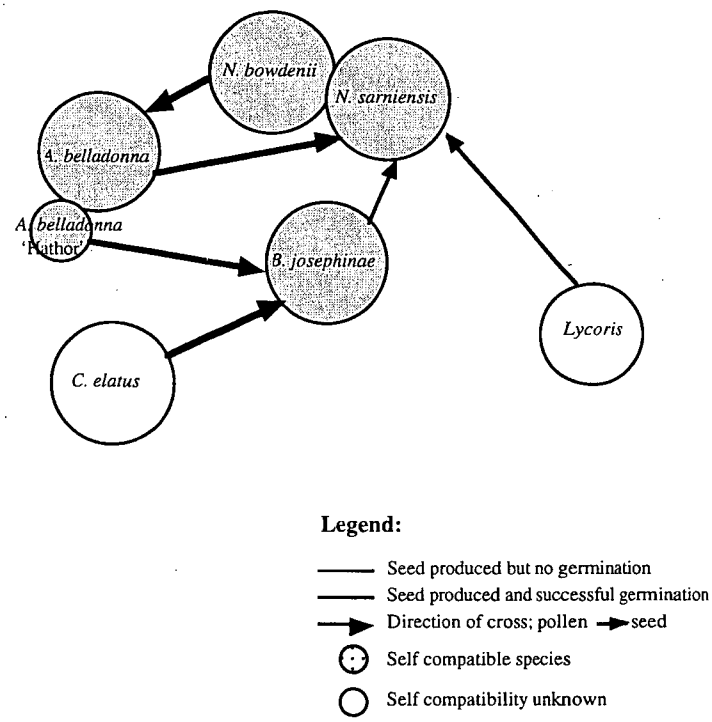


Figure 6.2: Summary of crossing relationships between genera held in the Channel Bulbs collection.

The cultivar *N. sarniensis* x 'Novelty' is purported to be a cross between *Nerine* and *Agapanthus* (Ammal and Bridgwater, 1951; Meninger, 1960) (Plate 6.3c). If this is the case, crosses involving *Nerine* x 'Novelty' are second generation bi-generic crosses. Two successful seedling producing crosses were achieved using x 'Novelty' as both seed and pollen parent (Table 6.7).

Cross
x 'Novelty' **X** x 'Corusca'
x 'Rosea' **X** x 'Novelty'

Table 6.7: Successful seed producing crosses, which are possibly bi-generic.

6.3.4 Identification of point of failure in hybrid crosses

Hybrid crosses which fail to produce viable progeny can be classified into three broad groups: (i) failure due to infertility of one or both parents, or inviability of the gametes at the time of crossing; (ii) breakdown of the fertilisation process prior to fertilisation occurring (pre-zygotic); or (iii) breakdown after fertilisation (post-zygotic) resulting in seed but no germination, breakdown of the hybrid prior to fertilisation or an infertile hybrid. As fertility problems have been discussed elsewhere (6.3.1; Chapter 3), crosses which fall into the first group will not be considered here.

6.3.4.1 Pre-zygotic barriers

Crosses at each level (inter-generic, inter-specific and intra-specific) exhibited pre-zygotic breakdown, as detected by lack of ovule swelling or seed production. This breakdown may have been due to: (i) pollen unable to germinate on the foreign stigma; (ii) pollen unable to penetrate the stigma; or (iii) retardation or cessation of pollen tube growth in the stigma, style or ovary.

Pollen tube growth

To determine at which point a barrier was occurring, progress of fertilisation was followed by microscopical observation of crosses at each hybridisation level: (i) the intra-specific cross *N. sarniensis* x 'Curiosity' **X** Self; (ii) the inter-specific cross *N. sarniensis* x 'Fothergilli major' **X** *N. masonorum*; and (iii) the inter-generic cross

Lycoris aurea **X** *N. sarniensis* x 'Fothergillii major'. These results permitted comparison to known compatible crosses (Table 6.8).

Cross	Stage of pollination	Number of pollen grains		Number of tubes in style	
		Total	Germinated	Top	Base
Intra-specific cross					
N. sarniensis x 'Curiosity' **X** Self	A+2 to A+3	55±5	38±7	18±2	5.5±1
Inter-specific cross					
N. sarniensis x 'Fothergillii major' **X** *N. masonorum*	A to A+2	194±58	108±47	-	5.4±3
Inter-generic cross					
Lycoris aurea **X** *N. sarniensis* x 'Fothergillii major'	A+1	4	4	0	0
Known compatible crosses					
N. bowdenii x 'Clone 63' **X** *N. bowdenii* x 'Clone 63'	A to A+3	8±2	5±3	0	0
N. bowdenii x 'Clone 63' **X** *N. bowdenii* x 'Clone 63'	A+4 to A+7	40±10	31±8	15±5	3±3
N. bowdenii x 'Clone 63' **X** *N. bowdenii* x 'Clone 63'	A+8 to A+11	105±24	78±13	52±27	28±6
N. sarniensis x 'Rosea' **X** *N. sarniensis* x 'Rosea'	A to A+3	25±8	18±6	12±4	2±1
N. sarniensis x 'Rosea' **X** *N. sarniensis* x 'Rosea'	A+4 to A+7	48±11	30±7	14±4	3±1
N. sarniensis x 'Rosea' **X** *N. sarniensis* x 'Rosea'	A+8 to A+11	188±31	143±24	81±14	24±4

Table 6.8: Pollen grains/tubes found in the pollen pathway.

In the intra-specific cross *N. sarniensis* x 'Curiosity' **X** Self, repeated three times, pollen was found to germinate, penetrate the stigma and grow down the style. The numbers of pollen tubes decreased with progress down the style, as is found in compatible crosses. Pollen tubes reaching the base of the style were low, but in concurrence with numbers reaching the base in known compatible crosses.

The inter-specific cross *N. sarniensis* x 'Fothergilli major' **X** *N. masonorum* was repeated eight times and in all cases: (i) pollen germinated on the foreign stigma; and (ii) pollen tubes penetrated the stigma, while only a small number grew to the base of the style. The number of tubes reduced with distance down the style, with only 5% of germinated pollen grains reaching the base (Table 6.9). This represents an average of three tubes per cross (less than 20% of those found growing into the top third of the style). The greatest reduction in the number of pollen tubes occurs at the stigma/style interface, a phenomenon that also occurs in compatible crosses when pollinations occur at a comparable time (A to A+3) (Table 6.8). Although numbers of pollen tubes reaching the base of the style are low, as with the intra-specific cross, they concur with numbers found in compatible crosses carried out at a similar stage (A to A+3).

Pollen of *N. sarniensis* x 'Fothergilli major' germinated on the stigma of *Lycoris aurea* (Table 6.8), however, the number of pollen grains adhering to the stigma was low. Pollen tubes of germinated pollen grains penetrated the stigma, however, their growth ceased at the top of the style.

Reciprocal Crosses

Reciprocal crosses were attempted to confirm reports that unidirectional crossability occurs in *Nerine* (Smee, 1984). However, limitations in plant material prevented all possible crosses. Consequently, a list of successful crosses is far from exhaustive. Nevertheless, thirteen intra-specific and one inter-specific cross were successful in both directions (Table 6.9).

Parents
N. sarniensis x 'Captain Dunne Cook' and *N. sarniensis* x 'Pink Fairy'
N. sarniensis x 'Chorister' and *N. sarniensis* x 'Indian Orange'
N. sarniensis x 'Corusca' and *N. sarniensis* x 'Cuckfield'
N. sarniensis x 'Corusca' and *N. sarniensis* x 'Kenilworth'
N. sarniensis x 'Corusca' and *N. sarniensis* x 'Rosea'
N. sarniensis x 'Imp' and *N. sarniensis* x 'Rosea'
N. sarniensis x 'Indian Orange' and *N. sarniensis* x 'Pink Opal'
N. sarniensis x 'Jill' and *N. sarniensis* x 'Donna'
N. sarniensis x 'Killi' and *N. sarniensis* x 'Virgo'
N. sarniensis x 'Lady Lucy' and *N. sarniensis* x 'Pink Fairy'
N. sarniensis x 'Mrs Cooper' and *N. sarniensis* x 'Western Sunset'
N. sarniensis x 'Rosea' and *N. bowdenii* x 'Clone 63'
N. sarniensis x 'Rosea' and *N. sarniensis* x 'Pink Fairy'
N. sarniensis x 'Sunset Frills' and *N. sarniensis* x 'Salmon Supreme'

Table 6.9: Successful reciprocal crosses.

Reciprocal crosses, which were unsuccessful are listed in Table A.6.10. A number of explanations may be given for the failure of unsuccessful reciprocal crosses: (i) the cross may have been attempted outside the fertile period (see Chapter 4); (ii) the parent may have reduced fertility as a pollen or seed parent (see Table A.6.8); or (iii) there was a barrier, either physical (*e.g.* disparate style lengths) or physiological (*e.g.* an incompatibility reaction). In consideration of the first two explanations, relevant fertility information is listed beside each unsuccessful cross (Table A.6.10).

Style measurements

To determine if relative style lengths of the seed and pollen parent had any bearing on the success of a hybrid cross, style lengths were measured at the stage of three open anthers (Fig. 6.3). Of successful seed producing crosses, 83% had the style length of the pollen parent greater than or equal to that of the seed parent.

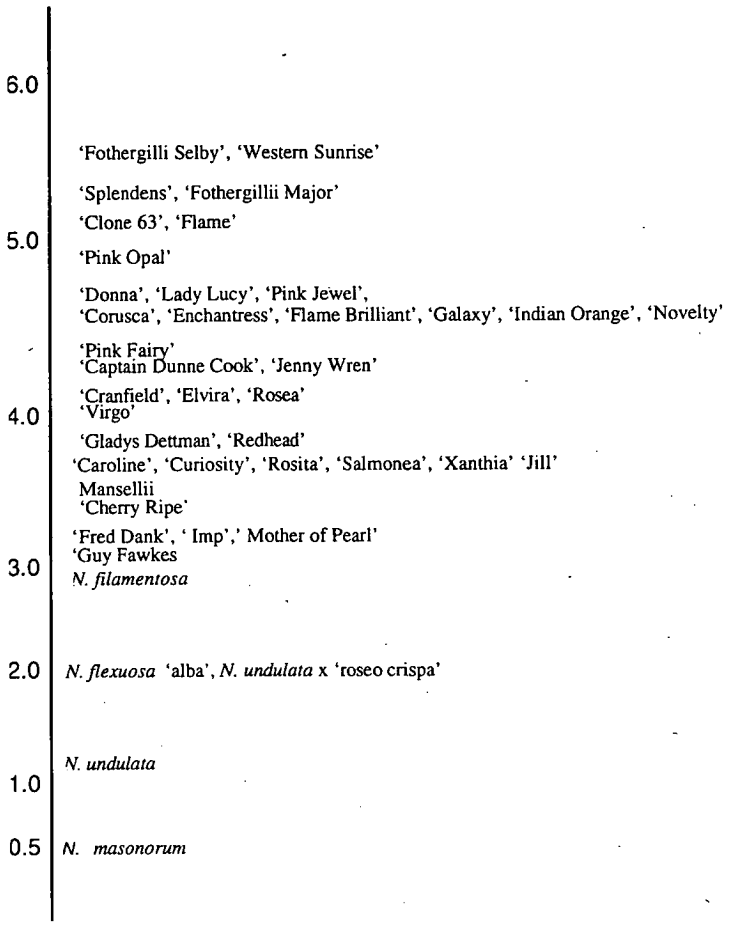


Figure 6.3: Style length (cm) in *Nerine* at the stage of three open anthers.

In the three inter-specific crosses that were successful in only one direction, the successful cross occurred when the style length of the pollen parent was greater than that of the seed parent. In each of these cases, the difference in style lengths was at least 2.5cm. This suggests that the physical barrier of style length may be important. However, in the failed intra-specific reciprocal crosses (where parents were known to be fertile), style length did not show a similar pattern. In fact, equal numbers of failed crosses had style lengths longer in pollen parents and seed parents. Differences between style lengths in intra-specific crosses were considerably smaller than the inter-specific cases, usually within 1cm of each other.

6.3.4.2 Post-zygotic barriers

Post-zygotic breakdown includes the scenarios where: (i) seeds have been produced but do not germinate (that may be due to a breakdown of the embryo, endosperm or both); (ii) hybrids which do not survive to flowering stage; and (iii) hybrids unable to produce fertile gametes.

Seed produced but unable to germinate

Seeds which did not germinate occurred in crosses at intra-specific, inter-specific and inter-generic levels (Table 6.10). The reason for seed inviability was not determined, but seeds where embryos have disintegrated have been found (Plate 6.3d). It is also possible parthenogenesis accounts for some of the ungerminated seed. The seeds originating from *N. flexuosa* 'alba' as seed parent are the most questionable. In particular, the crosses with *Nerine* x 'Cranfield' and *Nerine* x 'Old Rose' are doubtful, as both these pollen parents have been unsuccessful in producing seed in other crosses and pollen viability is low (as assessed *in vitro;* Table 6.2). Unfortunately, it is impossible to distinguish morphologically between sexually fertilised seed and parthenogenetic seed (see Chapter 5).

Intra-specific crosses	**Inter-specific crosses**
Nerine sarniensis	*N. bowdenii* x 'Clone 63'**X** *N. sarniensis* x 'Cpt. Dunne Cook'
x 'Brahms' **X** x' Jean O'Neill	*N. bowdenii* x 'Clone 63'**X** *N. sarniensis* x 'Cherry Ripe'
x 'Brahms' **X** x ' Jill'	*N. bowdenii* x 'Clone 63'**X** *N. sarniensis* x 'Eve'
x 'Caroline' **X** x 'Lady Lucy Hicks Beach'	*N. bowdenii* x 'Clone 63'**X** *N. sarniensis* x 'Mother of Pearl'
x 'Donna' **X** x 'Chorister'	*N. flexuosa* 'alba' **X** *N. sarniensis* x 'Cranfield'
x 'Donna' **X** x ' Lady Lucy Hicks Beach'	*N. flexuosa* 'alba' **X** *N. sarniensis* x 'Eve'
x 'Fothergillii major' **X** x 'Pink Opal'	*N. flexuosa* 'alba' **X** *N. sarniensis* x 'Pink Fairy'
x 'Fred Danks' **X** x 'Rosea'	*N. flexuosa* 'alba' **X** *N. sarniensis* x 'Pink Opal'
x 'Pink Fairy' **X** x 'Corusca'	*N. flexuosa* 'alba' **X** *Nerine* x 'Old Rose'
x 'Pink Fairy' **X** x 'Salmon Supreme'	**Inter-generic crosses**
x 'Pink Fairy' **X** x 'Splendens'	*Nerine sarniensis* x 'Rosea' **X** *Lycoris aurea*
x 'Rosea' **X** x 'Brahms'	*Nerine sarniensis* x 'Rosea' **X** *Brunsvigia josephinae*
x 'Splendens' **X** x 'Rosea'	*Nerine sarniensis* x 'Fothergillii major' **X** *Amaryllis belladonna*

Table 6.10 : Crosses producing seed that has not germinated.

Occurrence of hybrid sterility

The long period from fertilisation to flowering in *Nerine* means assessment of the fertility of generated hybrids during this study could not be completed. Nevertheless, it was possible to assess the fertility of hybrids present in the Channel Bulbs collection (see 6.3.2). Of the apparently infertile cultivars, five are inter-specific hybrids, while another is an inter-generic hybrid (Table 6.11). At least one of the inter-specific hybrids is known to be polyploid (*Nerine* x 'Aurora') (Meninger, 1960).

Nerine x 'Ancilla'*	*Nerine* x 'Old Rose' *
Nerine x 'Aurora'*°	*Nerine* x 'Salmonea'
Nerine x 'Cameo Beauty'	*N. sarniensis* x 'Baghdad'
Nerine x 'Fletcherii' #	*N. sarniensis* x 'Cranfield'
Nerine x 'Gilbert Errey'	*N. sarniensis* x 'Curiosity'
Nerine x 'Hera'*°	*N. sarniensis* x 'Elvira'
Nerine x 'Lucinda' *	*N. sarniensis* x 'Evelyn Humphries'
Nerine x 'Mansellii' *	*N. sarniensis* x 'Gladys Dettman'

Table 6.11: Apparently infertile hybrid cultivars in Channel Bulbs collection.
* Known inter-specific cross. ° Known polyploid. # Known inter-generic cross.

6.4 Discussion

The fertility status of 83 *Nerine* cultivars held in the Channel Bulbs collection has been established through controlled crosses and *in vitro* pollen germination. Several cultivars appear to be of reduced fertility, being unsuccessful (or rarely successful) as either pollen or seed parents (or both) in controlled crosses or as seed parents in open pollinations. This has been supported in many cases by *in vitro* germination data.

The use of pollen viability data from *in vitro* germination needs to be regarded with some caution as *in vitro* media can only approximate *in vivo* conditions (Rosen, 1971). Nevertheless, the data on pollen fertility collected from *in vitro* germination, microscopical observation of the proportion of empty pollen grains and from controlled crosses is consistent with only two exceptions. *N. sarniensis* x 'Cherry Ripe' was successfully used as a pollen parent, but failed to germinate *in vitro*. This was probably due to the use of pollen which was too old, and no longer viable, for the *in vitro* test. In the case of *N. sarniensis* x 'Optimist', *in vitro* germination rate was relatively high, yet no seed was set from *in vivo* pollination. Unfortunately, the cultivars chosen as seed parents were later determined as infertile. These included *N. sarniensis* x 'Latecomer', a normally fertile cultivar, which was damaged due to the effects of high summer temperatures in the season in which the pollination occurred (see Chapter 8). Therefore, the apparent anomalies in the *in vitro/in vivo* results for *N. sarniensis* x 'Cherry Ripe' and *N. sarniensis* x 'Optimist' are inconclusive. Thus it appears the *in vitro* germination protocol is a reasonably accurate test for

fertility in *Nerine*. This test has benefits over the seed set method, as it is easy and rapidly carried out in the laboratory, with results ready within two days.

Categorical determination of infertility, rather than low fertility, is very difficult, particularly when apparently normal gametophytes are present. Crosses must be performed under varying conditions and, where possible, microscopic observation of the fertilisation process used for confirmation of infertility in these cases. This was not possible in this study due to: (i) small numbers of representatives of each cultivar held (often only a single bulb, or bulbs which have been produced as offsets of a single bulb, and therefore are clones); and (ii) flowering of many cultivars occurring only once throughout the course of the study. These factors effectively reduced sample size. Small sample size increases the probability that the particular bulb is not representative of the cultivar as a whole. Sample size is even further reduced if detrimental environmental conditions have affected the bulb (*e.g.* those disturbing reproductive organ development, see Chapter 2). These issues may explain why some cultivars assessed as infertile in this study have previously been recorded as seed or pollen parents in other breeding programmes (Table A.6.7; Norris, 1992a; 1992b; Smithers, 1992a; 1992b).

When the fertility of cultivars has been assessed in two or more seasons, the effect of environmental factors becomes less problematic, and conclusions are given more weight. However, this does not overcome the possibility of a fertility problem restricted to that particular bulb. Nevertheless, as the bulbs held in the Channel Bulbs collection will form the basis of the Tasmanian breeding programme, it is the fertility status of these particular plants which is important.

Whether the observed cases of infertility or low fertility in the Channel Bulbs collection is the result of polyploidy, aneuploidy, hybrid (F_1) sterility or horticultural longevity, remains to be determined. Certainly, evidence exists that ploidy is a factor in *Nerine* fertility, with aneuploidy and polyploid reported in several *Nerine* cultivars (James and Addicott, 1941; Gouws, 1949; Ammal, 1951; Ammal and Bridgwater, 1951). Polyploidy and aneuploidy are also commonly found in the Amaryllidaceae. Genera with polyploid or aneuploid species or varieties include *Crinum* (Gouws, 1949), *Zephyranthes* (Flory, 1943), *Eucharis* (Meerow, 1984) and *Narcissus* (Hannibal, 1941).

Infertility, particularly of *Nerine* hybrids has been similarly reported by other breeders (*e.g.* Smee, 1984). However, the assessment of fertility of particular cultivars in this study does differ from that recorded in the literature (*e.g. Nerine* x 'Flame' and x 'Aurora'). This may be due to inaccuracies in naming of cultivars that have been purchased for the collection, or to the loss of fertility in these clones due to a long period in cultivation. The latter has been identified in some species where prolonged vegetative propagation has resulted in selection for fertility no longer being of importance. This can lead to increased cases of pollen and seed sterile cultivars, unbalanced chromosomes, chromosomal alterations, irregular meiosis and occurrence of several ploidy levels (Zohary, 1997).

Self-incompatible (SI) plants can recognise and arrest the growth of self pollen within the pistil, thereby promoting out-breeding and consequent genetic variation within a population (Newbigin *et al.*, 1993; Matton *et al.*, 1994). This phenomenon has not been reported in *Nerine*, and from these results it appears not to be present, at least in the cultivars tested. It is likely that *N. bowdenii*, *N. sarniensis* and the majority of their fertile cultivars are self-fertile. The cultivars where self pollinations were unsuccessful were most probably due to: (i) the low number of self pollinations performed; (ii) pollination not occurring during period of pistil receptivity; or (iii) low fertility of the hybrid. However, in two cultivars, *N. sarniensis* x 'Corusca' and *N. sarniensis* x 'Chorister', self pollination was attempted at a range of times relative to anthesis. Self pollinations were unsuccessful, yet these bulbs did set seed from other crosses. Hence further investigation, preferably microscopical observation, is warranted as SI could be occurring in these cultivars. This study has established that self-compatibility occurs in *N. flexuosa* 'alba', *N. masonorum* and *N. undulata.*

In *N. filamentosa*, SI may exist as seed was not obtained from self, cross or open pollinations in over 30 individuals. The inflorescences appeared to be fertile with mature, binucleate pollen and ovules with embryo sacs present. The pollen had low viability, so it is possible that a fertility problem exists. As all *N. filamentosa* bulbs held at Channel Bulbs were clones of a single mother plant, any infertility could be expected to be ubiquitous.

As found in *N. filamentosa*, the many *N. sarniensis* cultivars in which no viable seed was set either by self-pollination or in crosses, make conclusions difficult to reach without microscopic evidence (*e.g.* arrest of pollen tube growth, non-germination). This was not possible due to flowering occurring only once in many of these

cultivars. These difficulties were also true of other genera in the collection, with *Cyrtanthus elatus, Lycoris radiata* and *L. aurea* not setting seed by open pollination, crossing or selfing. However, both *L. radiata* [1] and *L. aurea* have been reported to set seed after selfing (Shii *et al.*, 1997), which supports the scenario of reduced fertility.

SI has been established in other amaryllids (Table A.6.11). Genera with species that are obligatory outbreeders are found in *Eucharis*, *Euchrosia*, *Hippeastrum*, *Rhodophiala* and *Urceolina*, where sibling sterility (inability to cross with different but related clones) has also been reported (Koopowitz, 1986). It has also been reported that at least one species of *Crinum* (*C. flaccidum*) is self-incompatible (Howell and Prakash, 1990).

In *Nerine*, sibling sterility does not appear to be operating with a high success rate in intra-specific crosses between fertile parents. Although not all intra-specific crosses were successful, other factors such as pollinations occurring outside the fertility window and use of pollen which was over two days old may be responsible for much of this lack of success. In other intra-specific crosses, seed was produced that did not germinate, suggesting the occurrence of a post-zygotic barrier. Whether this is due to the cultivars being too closely related is not yet known. The possibility of some of these seeds being parthenogenetic must also be considered as the frequency of occurrence in hybrid cultivars in unknown.

Inter-specific crosses are possible within the genus *Nerine*, and at least in some cases are achievable without using interventionist techniques. The crosses attempted in the present study have used only a small section of the genus, making broad generalisations regarding crossability difficult. The majority of inter-specific crosses reported in the literature have used species within the Section Bowdeniae (ex Traub, 1967), which includes *N. bowdenii*, *N. flexuosa*, *N. humilis*, *N. pudica* and *N. undulata*, however, *N. sarniensis* also figures prominently. The use of these species can be traced to the hardiness of the *N. bowdenii* group, as well as the colour and form of *N. sarniensis* inflorescences. Presently, it is not known whether other species have been used in hybridisation without success, or whether programmes reflect the inaccessibility of some species. Nevertheless, the most evident feature of the crossing polygon (Fig. 6.3) is the number of possible inter-specific combinations

[1] *L. radiata* growing at Channel Bulbs may be an infertile triploid (R. Crowden, pers. comm.).

that have yet to be achieved. Consequently, there is a great deal of yet untapped variation within the genus. In particular, there has been no use of Section Laticomae (although *N. krigei* has been reported in an inter-generic cross [McNeil, 1987]) while only a few representatives of Section Appendiculate having been used in inter-specific crosses.

Three of the successful inter-specific crossses from this study have not been previously reported in the literature, these are: *N. masonorum* **X** *N. bowdenii* x 'Pink Jewel'; *N. flexuosa* 'alba' **X** *N. bowdenii* x 'Clone 63'; and *N. flexuosa* 'alba' **X** *N. filamentosa.* Unfortunately, the resultant product of the cross *N. masonorum* **X** *N. bowdenii* x 'Pink Jewel' did not survive past the seedling stage.

Analysis of inter-specific crossing data has cast some doubt on the origin of crosses using *N. flexuosa* 'alba' as a seed parent, due to the common occurrence of parthenogenetic seed in this cultivar (see Chapter 4). Crosses between *N. flexuosa* 'alba' and the following pollen parents, *Nerine* x 'Cranfield', *Nerine* x 'Old Rose' and *N. filamentosa,* which are known to have pollen of very low viability (6.3.2), are certainly questionable. Therefore, these seedlings will need to be assessed following flowering, or by DNA analysis (see Chapter 10). In the case of the cross *N. flexuosa* 'alba' **X** *N. filamentosa,* the resultant seedling was albino, which may also be an indication of parthenogenesis (see Chapter 5). However, confirmation of the occurrence of albino seedlings from parthenogenetic seeds of *N. flexuosa* is not possible, as no known parthenogenetic seeds germinated in the course of this study.

Smee (1984), reported difficulty in achieving a successful cross from *N. flexuosa* **X** *N. bowdenii,* although the reciprocal was reported as being readily achieved. His explanation was the high degree of sterility of the seed parent. This has not been observed in the cultivar *N. flexuosa* 'alba'. Indeed, the reverse of Smee's (1984) observations have been found with pollen of *N. flexuosa* 'alba' being unable to effect fertilisation of any varieties other than itself, despite pollen having an apparently high viability when tested *in vitro* (see Chapter 3).

Several inter-specific crosses, between fertile species, did not produce seed. This raises the question of whether there may be some type of barrier, physical or biochemical, preventing viable pollen from reaching the ovules in some crosses.

Fluorescence microscopical observation of intra-stylar pollen tube growth can identify some crossing barriers. Unfortunately, due to flowering problems the opportunity for microscopical study of failed crosses was severely limited. In the inter-specific cross *N. sarniensis* x 'Fothergilli major' **X** *N. masonorum*, pollen germinated on the foreign stigma. Whether the observed 47% germination rate of *N. masonorum* pollen is below what would normally occur in an intra-specific cross is unknown (this was unable to be assessed due to non-flowering). Nevertheless, the numbers germinating are high and comparable with those observed in compatible crosses (see Chapter 3), suggesting that no barrier to inter-specific crossing occurs at the stigma level (*i.e.* pollen is able to hydrate and germinate on the foreign stigma).

Although numbers of tubes reduce with progress down the style, numbers are consistent with compatible crosses performed at the same stage. The major barrier to pollen tube growth occurs at the stigma/style barrier, which mirrors the situation in compatible crosses (see Chapter 3). Additionally, numbers proceeding to the style are not dissimilar to numbers in a compatible cross performed at the same time (*i.e* prior to the opening of the stigmatic lobes). The microscopical observations did not detect any abnormality in pollen tube growth, which along with retardation or cessation of pollen tube growth characterises inter-specific crosses in *Lilium* (Rosen, 1971). Thus, no evidence of a particular incompatibility response has been found. Nevertheless, there was no evidence that fertilisation had been effected (*i.e.* no ovular swelling), suggesting the barrier to fertilisation in this cross occurs either in the ovary or post-fertilisation.

It is possible that ovular incompatibility may be acting to prevent fertilisation, after pollen tube growth down the style. Barriers occurring at the ovary level are uncommon but have been found in species with hollow styles and binucleate pollen (Chichiriccò, 1990). These characteristics are found in *Nerine*. If ovular incompatibility was acting in the *N. sarniensis* x 'Fothergilli major' **X** *N. masonorum* cross, formation of seed would not be possible. However, the formation of a seed in this inter-specific cross has been possible using direct ovule pollination (see Chapter 7). As this seed did not germinate, it appears that fertilisation can occur, but a barrier exists post-fertilisation.

When crosses were only successful in one direction, chromosomal incongruency is an unlikely explanation for the failure, so barriers are likely to be pre-zygotic. It has been reported that reciprocal crosses have not always been possible between *Nerine*

species (Smee, 1984). This failure may be due to a physical constraint such as style length or a physiological phenomenon such as unilateral incompatibility. This phenomenon is also found in other bulbous species such as *Tulipa* (van Creij *et al.*, 1992) and *Lilium* (van Creij *et al.*, 1993). Its occurrence means that in order to obtain hybrids, it is not only selection of parents which is important, but also the cross direction.

In some genera, species with widely disparate style lengths have proven difficult to hybridise (Khush and Brar, 1992). This may also be occurring in failed reciprocal inter-specific crosses in *Nerine*, where styles of the maternal cultivar may be too long for the pollen tubes of shorter styled cultivars. This would be significant when using *N. flexuosa* 'alba' as the pollen parent. The pollen is known to be viable, yet out of 17 crosses where the seed parent was known to be fertile, only one seed was set. This seed only germinated in tissue culture, so it is possible it may have been parthenogenetic. Consequently, the possibility remains that the pollen tube is unable to grow the length of the longer style of the seed parent cultivar.

Numerous inter-specific and inter-generic crosses have been successful in only one direction (Strasburger, 1886, cited in Hogenboom, 1972; Harrison and Darby, 1955; McConchie *et al.*, 1994), a phenomenon referred to as unilateral incompatibility (UI) (Dahliwal, 1992). This phenomenon mostly occurs in crosses between SI and SC species with the pollen of SC species inhibited in the styles of SI species (Lewis and Crowe, 1958). The genetic control of this phenomenon has been attributed to the *S* locus, and hence is thought to be related to SI. However, this is somewhat contentious. Hogenboom (1972; 1973) believed any inter-specific incompatibility to be distinct from SI. A further complication to the understanding of UI is the occurrence of an identical reaction between two SI or two SC species (B. McClure, pers. comm.). Recent research into self-incompatibility and inter-specific incompatibility (including UI) has proposed that the two phenomena, at least partially, result from a common underlying mechanism (Trognitz and Schmiediche, 1993; Murfett *et al.*, 1996). Nevertheless, the action of the inhibition of pollen tube in many inter-specific crosses does resemble that seen in SI species. It has yet to be determined whether UI, as defined above, is acting in failed inter-specific reciprocal crosses in *Nerine*.

Inter-generic crosses between members of the tribe Amaryllideae, as defined by Snijman and Linder (1996), appear to have an excellent success rate (Figure 6.2).

Herein, five successful crosses using *Nerine* are described, with more reported in the literature (Table A.6.4). However, crosses using *Nerine* with other members of the Tribe Amaryllideae (*Boophone*, *Strumaria* and *Crossyne*) as well as many of the large genus of *Crinum*, have not been reported. This may be due to unsuccessful attempts, but is more likely to be attributable to the relative rarity of these genera, meaning little work has been done in this area. Members of these genera are currently being added to the Channel Bulbs collection, and will be used in future hybridising programmes.

The success of inter-generic crosses within the tribe suggests that the members of the Amaryllideae may be sufficiently genetically similar to prevent incongruency. In addition, the haploid chromosome number of 11 is considered to be consistent in most of the Amaryllidaceae (Goldblatt 1976; Gouws, 1949; Flory, 1977; Meerow, 1984), which is found in *Nerine* (James and Addicott, 1941; Gouws, 1949; Ammal, 1951), *Amaryllis* (Gouws, 1949), *Brunsvigia* (Gouws, 1949; Goldblatt, 1972) and *Boophone* (Gouws, 1949) as well as the closely related *Crinum* (Gouws, 1949; Flory, 1982).

The possibility of successful inter-generic crosses unlocks a huge pool of genetic variation for the development of improved horticultural varieties. However, as few crosses have been performed between *Nerine* and genera outside the Tribe Amaryllideae, it remains to be determined how wide a cross can be made. Production of a seed in the cross *N. sarniensis* x 'Rosea' **X** *Lycoris aurea* is greatly significant, as it offers the potential introduction of yellow tepal colour to *Nerine*. However, breakdown of the seed prior to germination indicates a barrier to hybridisation is acting at the post-zygotic level. It may therefore be necessary to use interventionist techniques to bring this cross to fruition (see Chapter 7).

The precise phylogenetic relationship of *Lycoris* to *Nerine* has varied with classification reviews. A general consensus places the two genera in different tribes, but variously in or out of the same sub-family (Traub, 1957, 1962a). Classification on chromosome number removed *Lycoris* [n = 12] from the same sub-family as *Nerine* [n = 11]. However, the base chromosome number of *Lycoris* is also subject to conjecture being reported as 6 - 9, 11, 12 and 15 (Bose, 1957; 1958a; 1958b; Traub, 1957; Meerow, 1984; Shii *et al.*, 1997).

Although a post-zygotic barrier appears to be operating in the inter-generic cross *N. sarniensis* x 'Rosea' **X** *Lycoris aurea*, a second cross using *L. aurea* as the seed parent and *N. sarniensis* x 'Fothergillii major' as the pollen parent, has been blocked by a pre-zygotic barrier. Pollen grains germinated and entered the stylar tissue, where tubes arrested. This suggests that the stigma does not provide a barrier to the cross but a physiological barrier appears to be acting in the style. Further microscopical observations of inter-generic crosses are necessary in order to confirm and extend this observation. Retardation or cessation of pollen tube growth, as seen in this case has been found in other inter-generic crosses (*e.g.* wheat and rye [Ladizinsky, 1992]).

The *Lycoris* pistil is reportedly weakly incongruent with related species and genera (Shii *et al.*, 1997), with pollen of some related genera able to germinate on *Lycoris* stigmata with pollen tube growth delayed or pollen tubes malforming once inside the style. However, some cases of fertilisation have been recorded (Coertze and Louw, 1990; van Brenk and Benschop, 1993; Shii *et al.*, 1997). This is consistent with the observations made during this study.

In addition to the cross *N. sarniensis* 'Rosea' **X** *L. aurea,* three other seed-producing inter-generic crosses succumbed to post-zygotic barriers, with seed unable to germinate. The inter-generic crosses that produced seedlings will need to be monitored, as it is possible that post-zygotic mechanisms may act in the future to cause hybrid breakdown or sterility.

The hybrid *Nerine* x 'Novelty' is interesting, as it is reported to be a cross between *Nerine sp.* and *Agapanthus sp. (i.e.* an inter-familial cross) (Meninger, 1960). Although this seems unlikely, and the possibility of an open pollinated or parthenogenetic seed would appear to be more likely, the form of the inflorescence in *Nerine* x 'Novelty' (Plate 6.3c), resembles the open form of *Agapanthus*. Additionally, the arrangement of stamens in two distinct rows, is unlike any other *Nerine* variety examined. This could represent an important breakthrough as a first generation hybrid, as *Nerine* x 'Novelty' is fertile, and has produced seed both as a seed and pollen parent.

Evidence of post fertilisation barriers manifests when seeds do not germinate; this occurred at all levels of hybridisation. Arrest or complete breakdown of embryo development is typical of many inter-specific and inter-generic crosses (Ladizinsky,

1992). This may be due to interactions between parental genomes in the embryo or abnormalities of the endosperm. In fact, it is the breakdown of the endosperm which is responsible for the failure of many inter-specific and inter-generic crosses (Williams and de Latour, 1980).

Whether seed breakdown in *Nerine* is due to endosperm or embryo breakdown has yet to be determined. The volume of endosperm in a mature seed is not large, which may mean it is the much larger integument that is important for embryo nutrition. This may also be the cause for non-germination of undersized seeds. However, there are numerous instances of seeds which appear normal but do not germinate. Clearly the integument in these seeds has increased as normal, pointing to a breakdown of either embryo or endosperm. Embryos which have ceased to grow at an early stage have been located in some of these non-germinating seeds. Small seeds, where integument has not developed, have germinated in tissue culture (see Chapter 7), which may mean the nutritional function of the integument is assumed by the artificial media.

Breakdown prior to reproductive maturity also appears to be operating in *Nerine*, again at all levels of hybridisation. This may manifest in seedlings disintegrating soon after germination, seedlings not surviving the first dormancy period, or hybrids surviving to flowering age but being infertile. In addition, it is recognised that some *Nerine* cultivars are better used either as seed or pollen parents (Smee, 1984).

Sterility in the F_1 generation is often due to chromosome imbalances (van Tuyl, 1997) and a number of hybrid *Nerine* cultivars exhibit polyploidy or aneuploidy (Table 6.12). This F_1 sterility is also found in *Lilium* hybrids (van Tuyl *et al.*, 1997). Nevertheless in *Nerine*, aneuploid or polyploid parents have produced progeny (*e.g. N. sarniensis* x 'Fothergillii major' in this study). Occasional seed set and limited pollen fertility has also been found in the tetraploid cultivar *Nerine* x 'Inchmery Kate' (Smee, 1984), and as a result several progeny have been produced. Whilst relying on low fertility parents to produce progeny is not feasible for crossing programmes, it is possible to use chemicals to double ploidy, and restore fertility to these plants (see Chapter 7). In addition, it is likely that crosses involving polyploid or aneuploid parents, such as *Nerine* x 'Inchmery Kate', *N. sarniensis* x 'Corusca' (2n=24) or *N. sarniensis* x 'Fothergilli major' (2n=33), would also exhibit aneuploidy or polyploidy. Again, restoration of fertility may be possible through chromosome doubling (see Chapter 7).

Diploid	Aneuploid	Triploid
x 'Fletcheri'	x 'Baghdad'	x 'Aurora'
x 'Lady Lucy Hicks Beach'	x 'Corusca'	x 'Dunkirk'
x 'Mansellii'	x 'Curiosity'	x 'Fothergillii major'
x 'Novelty'		x 'Hera'
x 'Mrs Cooper'		

Table 6.12: Chromosome numbers of hybrid clones in the Channel Bulbs collection (after Ammal, 1951; Ammal and Bridgwater, 1951).

6.5 Conclusion

The fertility of seven species and 76 cultivars has been assessed via *in vitro* pollen germination and controlled crossing, with evidence of low fertility or sterility in a number of hybrid cultivars. Self-compatibility has been established in five species and 23 cultivars.

A hybridisation programme operating at intra- and inter-specific and inter-generic levels was initiated, yielding seeds from 96 intra-specific, eleven inter-specific and eight inter-generic crosses. Identification of point of failure in unsuccessful hybrid crosses found pre- and post-zygotic barriers occurring at all hybridisation levels (intra-specific, inter-specific and inter-generic).

Plate 6.1a *Nerine sarniensis* x 'Fothergillii major'
Plate 6.1b *Nerine bowdenii* x 'Clone 63'
Plate 6.1c *Nerine flexuosa* 'alba'
Plate 6.1d *Nerine filamentosa*
Plate 6.1e *Nerine sarniensis*
Plate 6.1f *Nerine undulata*

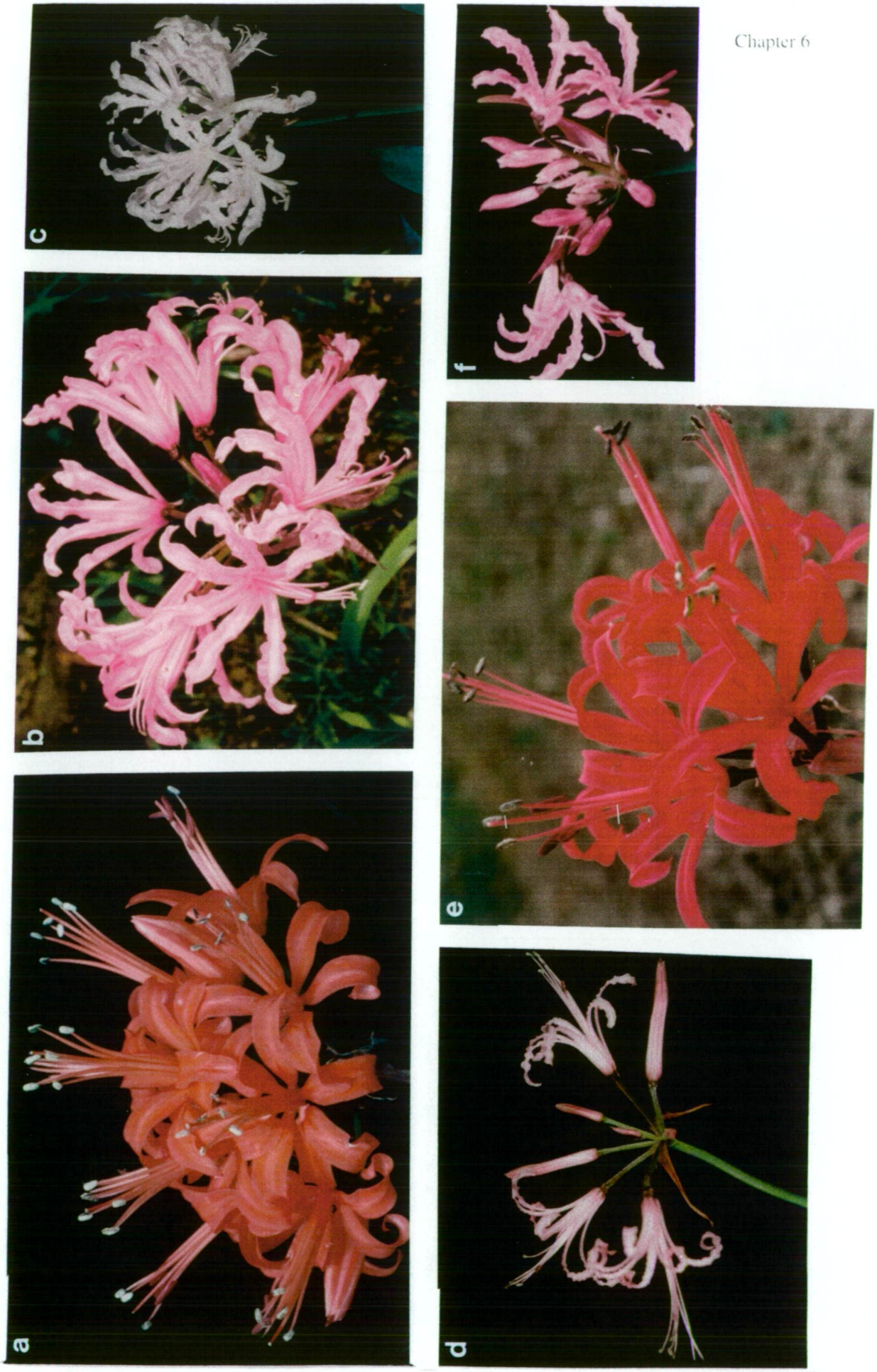

c
f
b
e
a
d

Plate 6.2a *Amaryllis belladonna* 'Hathor'
Plate 6.2b *Brunsvigia josephinae*
Plate 6.2c *Cyrtantus elatus*
Plate 6.2d *Lycoris aurea*
Plate 6.2e *Lycoris radiata*

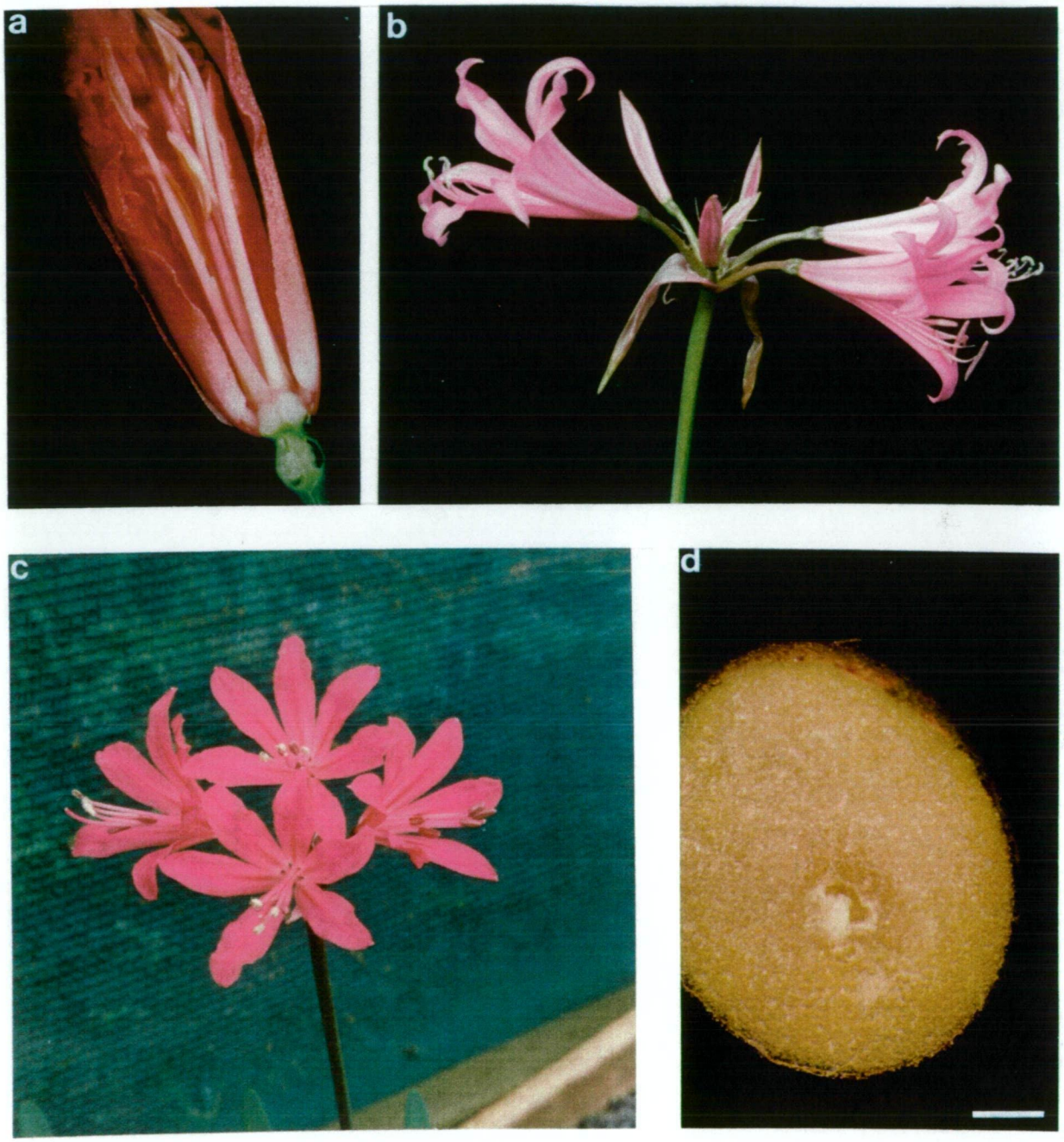

b
e
d
a
c

Plate 6.3a Floret of *Nerine* x 'Baghdad' showing empty anthers.

Plate 6.3b *Nerine* x 'Fletcherii'

Plate 6.3c *Nerine* x 'Novelty'

Plate 6.3d Cross section of *N. bowdenii* x 'Clone 63' seed with aborted embryo. Scale bar = 1cm.


a
b
c
d

Hybrid	Inter-specific Cross*	Hybridiser
Mitchamiae	*N. curvifolia* (*sarniensis*) **X** *N. undulata*	Herbert
Versicolor	*N. curvifolia* (*sarniensis*) **X** *N. undulata*	Herbert
Haylockii	*N. curvifolia* (*sarniensis*) **X** *N. pulchella* (*humilis*)	Herbert
Pulchello-undulata	*N. pulchella* (*humilis*) **X** *N. undulata*	Herbert
Spofforthiae	*N. venusta* (*sarniensis*) **X** *N. undulata*	Herbert
Pulchello-humilis	*N. pulchella* (*humilis*) **X** *N. humilis*	Herbert
Humilis-undulata	*N. humilis* **X** *N. undulata*	Herbert
Curvifolio-venusta	*N. curvifolia* (*sarniensis*) **X** *N. venusta* (*sarniensis*)	Herbert
Amabilis	*N. pudica* **X** *N. humilis*	O'B, L, C**
Cami	*N. curvifolia* (*sarniensis*) **X** *N. undulata*	O'B, L, C
Atrosanguinea	*N. Plantii* (*sarniensis*) **X** *N. flexuosa*	O'B, L, C
Cinnabarina	*N. fothergillii* (*sarniensis*) **X** *N. flexuosa*	O'B, L, C
O'Brien var carminata	*N. pudica* **X** *N. Plantii* (*sarniensis*)	O'B, L, C
O'Brien var caerulea	*N. pudica* **X** *N. Plantii* (*sarniensis*)	O'B, L, C
Erubescens	*N. flexuosa* **X** *N. undulata*	O'B, L, C
Elegans	*N. flexuosa* **X** *N. rosea* (*sarniensis*)	O'B, L, C
Meadowbankii	*N. sarniensis* **X** *N. fothergillii* (*sarniensis*)	O'B, L, C
Mansellii	*N. flexuosa* **X** *N. fothergillii* (*sarniensis*)	O'B, L, C
Roseo-crispa	*N. undulata* **X** *N. flexuosa*	O'B, L, C
Excellens	*N. flexuosa* **X** *N. humilis* major	O'B, L, C

Table A.6.1: Inter-specific hybrids listed in Baker. 1888. *Using present classification, some of these hybrids would be classed as intra-specific. **O'B, L, C = Messers O'Brien, Leichtlin, Cam and other experimenters. Reference: Baker, 1888.

Characteristic	Taxon
Scape over 1m	*N. angustifolia, N. bowdenii*
Pure white flowers	*N. rehmanii, N. pancratoides, N. bowdenii*, N. gibsonii*, N. flexuosa*, N. laticoma*, N. sarniensis**
Variegated flowers	*N. pudica, N. sarniensis*
Evergreen habit	*N. angustifolia, N. filifolia, N. flexuosa, N. masonorum*
Fast growth	*N. filifolia*
Well formed umbel	*N. sarniensis* (*e.g. N. sarniensis* x 'Fothergillii major')
Dwarf form	*N. masonorum, N. rehmanii*
Hardiness	*N. bowdenii*

Table A.6.2: Horticulturally desirable characteristics found in the genus *Nerine* (Baker, 1888; Traub, 1967; Norris, 1974; Brown, unpublished). (* varieties.)

Characteristic	Genera with ≥ 1 species showing characteristic
Scape over 1m	*Haemanthus, Brunsvigia, Crinum* (Harrison and Harrison, 1967); *Nerine* (Gallagher, 1967)
Multiple scapes	*Amaryllis* (McLaren, 1934); *Sternbergia* (Carter, 1934); *Hippeastrum Rhodophiala, Zephyranthes* (Harrison and Harrison, 1967); *Nerine* (Lehmilller, 1987); *Cyrtanthus* (Henry, 1942); *Ammocharis, Crinum* (Hannibal, 1955); *Narcissus* (Page and Olds, 1997)
Yellow corolla	*Narcissus, Childanthus* (Ulrich, 1934); *Cyrtanthus* (Dyer, 1936); *Amaryllis (Hipppeastrum), Lycoris* (Traub, 1941); *Anoiganthus, Clivea, Crinum, Cooperia, Spiloxene, Sternbergia, Zephyranthes* (Harrison and Harrison, 1967); *Stenomesson* (Meerow, 1987); *Narcissus* (Page and Olds, 1997)
Blue corolla	*Zephyranthes* (Traub, 1934); *Worsleya* (Traub, 1939)
Green corollas	*Stenomesson* sub genus *Callithauma* (Meerow, 1987)
White corollas	*Nerine* (Baker, 1888); *Crinum* (Page and Olds, 1997); *Narcissus* (Page and Olds, 1997)
Split corollas	*Hymenocalllis* (Hatch, 1987); *Narcissus* (Page and Olds, 1997)
Full radial umbel	*Crinum, Nerine* (Hatch, 1987)
Large flowers	*Hyline* (Traub, 1936); *Pancratium* (Traub, 1936); *Ammarylis, Sprekelia* (Harrison and Harrison, 1967)
Scent	*Eucharis* (Hayward, 1934); *Griffinia, Hyline* (Traub, 1936); *Cooperia* (Hughes, 1937); *Ammocharis* (Martley, 1939); *Childanthus, Hymenocallis* (Harrison and Harrison, 1967); *Gethyllis* (Stephens, 1939); *Narcissus* (Page and Olds, 1997)
Evergreen leaves	*Stenomesson* (Meerow, 1987); *Hymenocallis* (Hatch 1987); *Cyrtanthus* (Meerow, 1986); *Nerine* (Norris, 1974)
Ornamental foliage	*Eucharis, Hymenocallis* (Hatch, 1987); *Boophone* (Harridine, 1956)
Dwarf varieties	*Nerine, Crinum* (Flory, 1982); *Sprekelia* (Howard, 1976); *Hippeastrum* (Fesmire, 1970)
Fast growth	*Cyrtanthus* (Dyer, 1936); *N. filifolia* (R. Crowden, pers. comm.)

Table A.6.3: Horticulturally desirable characteristics found in the Amaryllidaceae.

Inter-generic Cross	Hybridiser	Reference
A. balladonna x *Nerine bowdenii*	Fletcher	Meninger, 1960; Doutt, 1994
A. belladonna x *Nerine angustifolia*	Van Tubergen	Doutt, 1994
A. belladonna x *Nerine sarniensis*	Van Tubergen	Gallagher, 1967
A. belladonna x *Nerine sp.*	Coertze and Louw	Coertze and Louw, 1990; Smithers 1990
A. belladonna x *Brunsvigia sp.*	Parker, Bidwell, Coertze and Louw	Krelage, 1938; Coertze and Louw 1990
A. belladonna x *B. grandiflora*	Bidwell	Gallagher, 1967
A. belladonna x *B.orientalis*	Loubser	Traub, 1949
A. belladonna x *B. josephinae*	Bidwell (1870)	Traub, 1949
A. belladonna x *B. josephinae* x*B. gigantea*	Parker	Doutt, 1994
A. belladonna x *Brunsdonna bidwellii* *Brunsdonna bidwellii* x *A. belladonna*	Not known (backcross)	Hannibal 1980
A. belladonna x *Clivia miniata*	Coertze and Louw	Coertze and Louw 1990
A. belladonna x *Crinum sp.*	Parker	Hannibal, 1942
A. belladonna x *Crinum (4n)*	Hannibal	Hannibal, 1942
A .belladonna x *Crinum bulbispermum*	Coertze and Louw	Coertze and Louw 1990
A. belladonna x *Crinum moorei*	Hannibal; Coertze and Louw	Traub, 1961a; Coertze and Louw 1990
A. belladonna x *Crinum powellii*	Howard	Hannibal, 1943
A. belladonna x *Crinum americanum*	Burbank	Hannibal, 1943
A. belladonna x *Crinum amabile*	Not known	Orpet, 1942
A. belladonna x *Sprekelia vittata*	Burbank	Hannibal, 1943
Brunsvigia josephinae x *A. belladonna*	Van Tubergen	Traub and Hannibal, 1961
Brunsvigia sp. x *A. belladonna*	Hannibal	Hannibal, 1942
Brunsvigia parkeri x *Crinum moorei*	Not known	Traub, 1962b; Anderson, 1961
Brunsvigia marginata x *A. belladonna*	van der Walt	Doutt, 1994
Brunsvigia marginata x *Nerine sarniensis*	Loubser	Doutt, 1994
Brunsvigia orientalis x *A.belladonna*	Hannibal	Doutt, 1994
Brunsvigia major x *Crinum moorei*	Not known	Traub, 1962b
Brunsvigia parkeri alba x *Crinum moorei roseum*	Traub	Traub, 1962b
Nerine sarniensis x *Brunsvigia minor*	Coertze and Louw	Coertze and Louw, 1990
Nerine humilis x *Brunsvigia appendiculata*	Norris	Norris, 1992b
Nerine bowdenii x *Lycoris sprengeri*	Not known	Forbes, 1973
Nerine bowdenii x *A. belladonna*	Bidwell	Gallagher, 1967
Nerine angustifolia x *A. belladonna*	Coertze and Louw	Coertze and Louw, 1990
Nerine sp. x *Agapanthus sp.*	Not known	Meninger, 1960; McNeil, 1987
Nerine krigei x *Brunsvigia sp.*	Not known	McNeil, 1987
Nerine sp. x *Brunsvigia sp.*	Meyer	Hardman, 1985
Nerine sarniensis x *Clivia miniata*	Orpet, Coertze and Louw	Hannibal, 1942; Coertze and Louw, 1990
Crinum moorei x *A.belladonna*	Traub	Traub, 1961a; Doutt, 1994
Crinum moorei x *Brunsvigia rosea* var minor	Not known	Norton and Ballard, 1952
Crinum sp. x *A. belladonna*	Not known	Hannibal, 1980
Crinum sp. x *A. burbank*	Burbank	Howard, 1942
Crinum (4n) x *A. belladonna* x *A. multiflora*	Not known	Hannibal, 1980

Table A.6.4: Documented inter-generic crosses of members of Tribe Amaryllideae (Amaryllidaceae). The nomenclature of *A. belladonna* has been inconistent (see Appendix A.6.12), therefore, crosses using *A. belladonna* may be recorded under a synonym.

Cross	Cross
N. bowdenii **X** *A. belladonna* 'Mithras' 'Pavlov' *A. belladonna* **X** *N. bowdenii* 'Fletcherii' *A. belladonna* **X** *Nerine sp.* 'Zwanenburg Beauty'	*Nerine sp.* **X** *Agapanthus sp.* 'Novelty' *N. humilis* **X** *Brunsvigia appendiculata* 'Expo'

Table A.6.5: Named inter-generic *Nerine* hybrids (for reference list refer to Appendix A: Review of *Nerine* cultivars).

Cross	Cross
N. bowdenii **X** *N. sarniensis* 'Dover' 'Exonia' 'Goya' 'Jose' 'Kara' 'Kingship' 'Kitanga' 'Kyanga' 'Laramie' 'Largo' 'Nabob' 'Natalie' 'Neil' 'Nevis' 'Oberon' 'Palamo' 'Pamir' 'Panam' *N. sarniensis* **X** *N. bowdenii* 'Aurora' 'Hera' *N. sarniensis* **X** *N. undulata* 'Cami' 'Hawaii' 'Haylockii' 'Mitchamiae' 'Spofforthiae' 'Versicolour' *N. sarniensis* **X** *N. pudica* 'Caerulea' 'Carminata' 'Stricklandii' *N. pudica* **X** *N. sarniensis* 'Karma' 'Karoo' 'Latu' 'Nicolai' 'O'Brienii' 'Phoebe' 'Zoroaster' *N. filifolia* **X** *N. sarniensis* 'Alma Moldenke' 'Chameleon' 'Joker' *N. gibsonii* **X** *N. sarniensis* 'La Lune' *N. undulata* **X** *N. flexuosa* 'Roseo-crispa' *N. flexuosa* **X** *N. undulata* 'Erubescens' 'Pulchello-undulata' *N. flexuosa* 'alba' **X** *N. sarniensis* 'Ancilla' 'Bettina' *N. angustifolia* **X** *N. sarniensis* Un-named (Coertze and Louw, 1990)	*N. bowdenii* **X** *N. pudica* 'Kosino' 'Manica' *N. bowdenii* **X** *N. flexuosa* 'Kymina' *N. bowdenii* **X** *N. humilis* 'Anne Rolls' 'Karen' *N. bowdenii* **X** *N. filifolia* Un-named (Cowlishaw, 1935) *N. sarniensis* **X** *N. flexuosa* 'Atrosanguinea' 'Kola' 'Konak' 'Mandele' *N. flexuosa* **X** *N. sarniensis* 'Elegans' 'Elegans alba' 'Elegans carminata' 'Mansellii' 'Queen of Sheba' *N. flexuosa* **X** *N. pudica* 'Flexudica' *N. flexuosa* **X** *N. humilis* 'Excellens' *N. humilis* **X** *N. sarniensis* 'Natasha' *N. humilis* **X** *N. undulata* 'humilis-undulata' *N. platypetala* **X** *N. angustifolia* 'Mandi' *N. pudica* **X** *N. humilis* 'Amabilis'

Table A.6.6: Inter-specific *Nerine* hybrids (for reference list refer to Appendix A: *Nerine* hybrid cultivars).

Species/Cultivar	% viability *in vitro*	viable *in vivo*
Nerine sarniensis x 'Ancilla'	nd	No
Nerine sarniensis x 'Angela Limerick'	+++	Yes
Nerine x 'Aurora'	nd	No #
Nerine sarniensis x 'Brahms'	-	Yes
Nerine bowdenii x 'Clone 63'	+++	Yes
Nerine sarniensis x 'Cameo Beauty'	+	No
Nerine sarniensis x 'Captain Dunne Cook'	-	Yes
Nerine sarniensis x 'Cherry Ripe'	nd*	Yes
Nerine sarniensis x 'Chorister'	-	Yes
Nerine sarniensis x 'Corusca'	+++	Yes
Nerine sarniensis x 'Cranfield'	-	Yes**
Nerine sarniensis x 'Cuckfield'	+	Yes
Nerine sarniensis x 'Curiosity'	+	No #
Nerine sarniensis x 'Cynthia Chance'	-	Yes
Nerine sarniensis x 'Donna'	-	Yes
Nerine sarniensis x 'Early Snow'	-	Yes
Nerine sarniensis x 'Enchantress'	+++	Yes
Nerine sarniensis x 'Eve'	-	Yes**
Nerine sarniensis x 'Fothergilli Major'	++	Yes
Nerine sarniensis x 'Flame'	nd	No #
Nerine sarniensis x 'Fred Dank'	-	Yes
Nerine sarniensis x 'Galaxy'	-	Yes
Nerine sarniensis x 'Gold Dust'	-	Yes
Nerine sarniensis x 'Imp'	-	Yes
Nerine sarniensis x 'Indian Orange'	-	Yes
Nerine sarniensis x 'Jean O'Neill'	-	Yes
Nerine sarniensis x 'Jenny Wren'	+	No
Nerine sarniensis x 'Jill'	+++	Yes
Nerine sarniensis x 'Kenilworth'	+	Yes
Nerine sarniensis x 'Killi'	-	Yes
Nerine sarniensis x 'Lady Lucy'	-	Yes
Nerine sarniensis x 'Latecomer'	+++	Yes
Nerine x 'Lucinda'	nd	No
Nerine sarniensis x 'Magenta Princess'	-	Yes
Nerine sarniensis x 'Mother of Pearl'	-	Yes
Nerine sarniensis x 'Mrs Bromley'	-	Yes
Nerine sarniensis x 'Mrs Cooper'	-	Yes
Nerine x 'Novelty'	+	Yes
Nerine x 'Old Rose'	+	Yes**
Nerine sarniensis x 'Optimist'***	++	No #
Nerine sarniensis x 'Pink Distinction'	-	Yes
Nerine sarniensis x 'Pink Fairy'	-	Yes
Nerine bowdenii x 'Pink Jewel'	-	Yes
Nerine sarniensis x 'Pink Opal'	-	Yes
Nerine sarniensis x 'Red Head'	+	Yes
Nerine sarniensis x 'Rosea'	+++	Yes
Nerine sarniensis x 'Rushmere Star'	nd*	No

Species/cultivar	% viability *in vitro*	viable *in vivo*
Nerine x 'Salmonea'	nd	No
Nerine sarniensis x 'Salmon Supreme'	-	Yes
Nerine sarniensis x 'Shelagh Mulholland'	-	Yes
Nerine sarniensis x 'Sunset Falls'	+++	Yes
Nerine sarniensis x 'Virgo'	-	Yes
Nerine sarniensis x 'Western Sunrise'	-	Yes
Nerine sarniensis x 'White Dove'	-	Yes
Nerine bowdenii x 'Winter Cheer'	-	Yes**
Nerine sarniensis x 'Xanthia'	-	Yes
Nerine filamentosa	-	Yes**
Nerine flexuosa alba	++	Yes
Nerine masonorum	++	Yes
Nerine undulata	++	Yes
Nerine undulata x 'Roseo-crispa'	-	Yes
Amaryllis belladonna	+++	Yes
Brunsvigia josephinae	+	Yes
Crinum powelli	-	No

Table A.6.7: Pollen viability results from *in vitro* germination and controlled crossing.

Key:
+++ = 60 % +
++ = 20 - 60%
+ = 1 - 20%
nd = no germination detected
- = not assessed

* pollen may have been too old
** seed not germinated, probably parthenogenetic
*** only flowered in 1997, may have been climate affected

Has been recorded as a pollen parent by Sir Peter Smithers (Breeding Records, 1992).

Channel Bulbs *Nerine* Collection
Fertility Table

Species/Variety	Ploidy	Pollen *in vitro*	Viability *in vivo*	Seed	S C
Nerine bowdenii	2n = 22	High	Yes	Yes	Yes
Nerine corusca	2n = 24	High	Yes	Yes	No (4)
Nerine filamentosa	2n = 22	ND	No	No	Inf?
Nerine flexuosa alba	2n = 22	High	Yes	Yes	Yes
Nerine humilis	2n = 22	n/a	n/a	Yes	n/a
Nerine masonorum	2n = 22	n/a	Yes	Yes	Yes
Nerine undulata	2n = 22	n/a	Yes	Yes	Yes
Named hybrid					
x Afterglow		n/a	No	No	Inf?
x Ancilla		ND	No	No	Inf?
x Angela Limerick		High	Yes	Yes	Yes
x Aurora	2n = 33	ND	No	No	Inf?
x Baghdad	2n = 24	n/a	No	No	Inf?
x Brahms		n/a	Yes	Yes	Yes
x Cameo Beauty		Low	No	No	Inf?
x Canasta		n/a	Yes	Yes	No (1)
x Captain Dunne Cook		n/a	Yes	Yes	No (2)
x Caroline	2n = 33	n/a	n/a	Yes	No (1)
x Cherry Ripe		ND	Yes	Yes	No (3)
x Chorister		n/a	Yes	Yes	No (3)
x 'Clone 63'		High	Yes	Yes	Yes
x Corusca	2n = 24	n/a	Yes	Yes	No (4)
x Cranfield		n/a	Yes **	No	Inf?
x Crispa		n/a	No	No	Inf?
x Cuckfield		Low	Yes	Yes	Yes
x Curiosity	2n = 24	Low	Yes	No	Inf?
x Cynthia Chance		n/a	Yes	Yes	Yes
x Donna		n/a	Yes	Yes	No (3)
x Early Snow		n/a	Yes	Yes	No (2)
x Elvira		n/a	No	No	Inf?
x Enchantress		High	Yes	Yes	No (1)
x Eve		n/a	Yes	Yes	Yes
x Evelyn Humphries		n/a	No	No	Inf?
x Flame		ND	Yes	Yes	n/a

Species/Variety	Ploidy	Pollen	Viability	Seed	S C
		in vitro	*in vivo*		
x Flame Brilliant		n/a	No	Yes	n/a
x Fletcherii		n/a	No	No	Inf?
x Fothergillii major	2n = 33	High	Yes	Yes	Yes
x Fred Danks		n/a	Yes	Yes	Yes
x Galaxy		n/a	Yes	No	Inf?
x Gladys Dettman		n/a	No	No	Inf?
x Gilbert Errey		n/a	No	No	Inf?
x Gold Dust		n/a	Yes	Yes	Yes
x Guy Fawkes		n/a	No	No	Inf?
x Hera	2n = 33	n/a	No	No	Inf?
x Imp		n/a	Yes	Yes	No (1)
x Indian Orange		n/a	Yes	Yes	Yes
x Jean O'Neill		n/a	Yes	Yes	Yes
x Jenny Wren		Low	No	No	Inf?
x Jill		High	Yes	Yes	Yes
x Judith		n/a	No	No	Inf?
x Kenilworth		n/a	Yes	Yes	Yes
x Killi		n/a	Yes	Yes	No (3)
x Lady Lucy Hicks Beach	2n = 22	n/a	Yes	Yes	No (1)
x Late Comer		High	Yes	Yes	No (2)
x Lucinda		ND	No	No	Inf?
x Magenta Princess		n/a	Yes	Yes	n/a
x Mansellii		n/a	No	No	Inf?
x Mother of Pearl		n/a	Yes	Yes	No (1)
x Mrs Bromley		n/a	Yes	Yes	Yes
x Mrs Cooper	2n = 22	n/a	Yes	Yes	No (1)
x Novelty	2n = 22	Low	Yes	Yes	No (2)
x Old Rose		Low	Yes	No	Inf?
x Optimist		High	No*	No	No (1)
x Pink Distinction		n/a	Yes	No	No (4)
x Pink Fairy		n/a	Yes	Yes	Yes
x Pink Jewel (Aust)		n/a	Yes	Yes	No (4)
x Pink Opal		n/a	Yes	Yes	Yes
x Red Head		Low	Yes	Yes	ND
x Rosamund Elwes		n/a	Yes	Yes	Yes
x Rosea (Aust)		High	Yes	Yes	Yes
x Roseo crispa		n/a	Yes	Yes	Yes

Species/Variety	Ploidy	Pollen Viability		Seed	S C
		in vitro	*in vivo*		
x Rosita		n/a	No	No	Inf?
x Rushmere Star		ND	No	No	Inf?
x Salmon Supreme		n/a	Yes	Yes	No (1)
x Salmonea		ND	No	No	Inf?
x Shelagh Mulholland		n/a	Yes	Yes	Yes
x Snow Maiden		n/a	Yes	No	n/a
x Splendens		n/a	Yes	Yes	No (3)
x Sunset Falls		n/a	Yes	Yes	Yes
x Virgo		n/a	Yes	No	n/a
x Western Sunset		n/a	Yes	Yes	No (3)
x White Dove		n/a	Yes	Yes	Yes
x Winter Cheer		n/a	Yes	Yes	Yes
x Xanthia		n/a	Yes	Yes	Yes

Table A.6.8: Fertility of species and cultivars in the Channel Bulbs *Nerine* Collection.

Key: SC = Self-compatible
n/a = Not assessed
Yes = Fertile, seed produced from cross
No = No seed produced from cross
ND = No germination detected (*in vitro*)
* = Pollen tested only on infertile cultivars, information inconclusive
** = Seed produced may have been parthenocarpic, information inconclusive
Inf? = Possibly infertile
( ) = Number of crosses performed

Reference for chromosome numbers: Ammal (1951); Ammal and Bridgwater (1951).

Cross: *N. sarniensis* x	Cross: *N. sarniensis* x
'Angela Limerick' X 'Corusca'	'Jill' X 'Pink Fairy'
'Angela Limerick' X 'Early Snow'	'Jill' X 'Western Sunset'
'Angela Limerick' X 'Enchantress'	'Jill' X 'Xanthia'
'Angela Limerick' X 'Jean O'Neill'	'Kenilworth' X 'Corusca'
'Angela Limerick' X 'Jill'	'Kenilworth' X 'Cuckfiled'
'Angela Limerick' X 'Kenilworth'	'Kenilworth' X 'Jill'
'Angela Limerick' X 'Pink Distinction'	'Killi' X 'Sunset Frills'
'Angela Limerick' X 'White Dove'	'Killi' X 'Virgo'
'Brahms' X 'Lady Lucy'	'Lady Lucy' X 'Pink Fairy'
'Captain Dunne Cook' X 'Corusca' *	'Latecomer' X 'Rosea'
'Captain Dunne Cook' X 'Pink Fairy'	'Magenta Princess' X 'Latecomer'
'Captain Dunne Cook' X 'Rosea'	'Magenta Princess' X 'Rosea'
'Chorister' X 'Galaxy'	'Mother of Pearl' X 'Chorister'
'Chorister' X 'Indian Orange'	'Mrs Bromley' X 'Cynthia Chance'
'Corusca' X 'Cuckfield'	'Mrs Cooper' X 'Mother of Pearl'
'Corusca' X 'Kenilworth'	'Mrs Cooper' X 'Pink Fairy' *
'Corusca' X 'Rosea'	'Mrs Cooper' X 'Western Sunset'
'Cuckfield' X 'Kenilworth'	'Mrs Cooper' X 'Xanthia' *
'Cuckfield' X 'Pink Distinction'	'Pink Fairy' X 'Captain Dunne Cook'
'Early Snow' X 'Brahms'	'Pink Fairy' X 'Cherry Ripe'
'Early Snow' X 'Lady Lucy Hicks Beach'	'Pink Fairy' X 'Donna'
'Enchantress' X 'Corusca'	'Pink Fairy' X 'Lady Lucy'
'Enchantress' X 'Rosea'	'Pink Fairy' X 'Mrs Cooper'
'Eve' X 'Jill'	'Pink Fairy' X 'Rosea'
'Flame' X 'Fothergillii' *	'Pink Opal' X 'Rose Princess'
'Flame' X 'Fothergillii major' *	'Redhead' X 'Corusca'
'Flame Brilliant' X 'Latecomer'	'Redhead' X 'Rosea'
'Flame Brilliant' X 'Mother of Pearl'	'Rosea' X 'Corusca'
'Flame Brilliant' X 'Salmon Supreme'	'Rosea' X 'Cynthia Chance'
'Flame Brilliant' X 'Snow Maiden'	'Rosea' X 'Curiosity'
'Fothergilli major' X 'Redhead'	'Rosea' X 'Imp'
'Fothergilli major' X 'Rosea'	'Rosea' X 'Jill'
'Fred Danks' X 'Corusca'	'Rosea' X 'Lady Lucy'
'Gold Dust' X 'Brahms'	'Rosea' X 'Magenta Princess'
'Gold Dust' X 'Chorister'	'Rosea' X 'Pink Distinction'
'Gold Dust' X 'Galaxy'	'Rosea' X 'Pink Fairy'
'Gold Dust' X 'White Dove'	'Splendens' X 'Angela Limerick'
'Imp' X 'Rosea'	'Splendens' X 'Killi'
'Indian Orange' X 'Chorister'	'Sunset Frills' X 'Donna'
'Indian Orange' X 'Pink Opal'	'Sunset Frills' X 'Imp'
'Jean O'Neill' X 'Angela Limerick'	'Sunset Frills' X 'Lady Lucy'
'Jean O'Neill' X 'Killi'	'Sunset Frills' X 'Salmon Supreme'
'Jean O'Neill' X 'Lady Lucy'	'Salmon Supreme' X 'Captain Dunne Cook'
'Jean O'Neill' X 'Sunset Frills'	'Salmon Supreme' X 'Donna'
'Jean O'Neill' X 'White Dove'	'Salmon Supreme' X 'Sunset Falls'
'Jill' X 'Corusca' *	'Western Sunset' X 'Mrs Cooper'
'Jill' X 'Donna'	'Xanthia' X 'Chorister'

Cross: *N. sarniensis* x	Cross: *N. sarniensis* x
'Jill' **X** 'Early Snow'	'Xanthia' **X** 'Donna'
'Jill' **X** 'Jean O'Neill'	'Xanthia' **X** 'Lady Lucy' *
'Jill' **X** 'Killi'	'Xanthia' **X** 'Pink Fairy'
'Jill' **X** 'Mother of Pearl'	'Xanthia' **X** 'Rosea'
'Jill' **X** 'Mrs Cooper'	

Table A.6.9: Intra-specific crosses of *N. sarniensis* (first named is seed parent).
* Seedling germination via tissue culture (see Chapter 7).

Cross	Fertility information
Inter-specific crosses	
N. bowdenii x 'Clone 63'* **X** *N. flexuosa* 'alba'*	Has set seed at time of pollination. Pollen viable.
N. bowdenii x 'Pink Jewel'* **X** *N. masonorum*'*	Has set seed. Pollen viable.
N. sarniensis x 'Eve'* **X** *N. flexuosa* 'alba'*	Has set seed. Pollen viable.
Intra-specific crosses (*N. sarniensis* x)	
'Brahms'* **X** 'Gold Dust'*	Has set seed at time of pollination. Pollen viable.
'Captain Dunne Cook' **X** 'Salmon Supreme'	Has set seed at time of pollination. Pollen viable.
'Chery Ripe' **X** 'Rosea'*	No seed set in 'Cherry Ripe'. Pollen viable.
'Chery Ripe' **X** 'Pink Fairy'*	See above.
'Corusca'* **X** 'Fred Dank'*	Has set seed at time of pollination. Pollen viable.
'Corusca'* **X** 'Novelty'	Has set seed at time of pollination. Pollen viable.
'Corusca'* **X** 'Pink Fairy'*	Has set seed at time of pollination. Pollen viable.
'Corusca'* **X** 'Red Head'	Has set seed at time of pollination. Pollen viable, but fertility reduced.
'Curiosity' **X** 'Rosea'*	No seed set in 'Curiosity'. Pollen viable.
'Donna' **X** 'Jill'*	Seed set in 'Donna', but has not germinated - may be reduced fertility. Pollen viable.
'Donna' **X** 'Xanthia'*	See above.
'Galaxy' **X** 'Gold Dust'*	No seed set in 'Galaxy'. Pollen viable.
'Jill'* **X** 'Rosea'*	Has set seed at time of pollination. Pollen viable. (Mulitple crosses.)
'Lady Lucy' **X** 'Rosea'*	Only other seed at A+7, pollination (at A) may have been too soon. Pollen viable.
'Mrs Cooper' **X** 'Pink Fairy'*	Has set seed at time of pollination. Pollen viable.
'Novelty' **X** 'Rosea'*	Has set seed at time of pollination. Pollen viable.
'Pink Distinction' **X** 'Cuckfield'*	No seed set in 'Pink Distinction'. Pollen viable.
'Pink Distinction' **X** 'Rosea'*	No seed set in 'Pink Distinction'. Has set seed
'Red Head'* **X** 'Fothergillii major'*	Has set seed at time of pollination. Pollen viable, but fertility reduced.
'Rosea'* **X** 'Splendens'	Has set seed at time of pollination. Pollen viability unknown.
'Rosea'* **X** 'Fothergillii major'*	Has set seed at time of pollination. Pollen low fertility. (Multiple crosses.)

Cross	Fertility information
'Rosea'* **X** 'Red Head'*	Has set seed at time of pollination. Pollen viable, but fertility reduced. May have been due to heat damaging inflorescences in 1997.
'Splendens' **X** 'Pink Fairy'*	Has set seed. Pollen viable. May have been too early.
'Xanthia'* **X** 'Brahms'*	Has set seed at time of pollination. Pollen viable.

Table A.6.10: Fertility information from failed reciprocal crosses. * Self compatible cultivar.

Genus	Reference
Boophane	Koopowitz, 1986
Brunsvigia	Koopowitz, 1986
Clivia	Koopowitz, 1986
Crinum	Howell and Prakash, 1990
Cyrtanthus	Koopowitz, 1986
Eucharis	Koopowitz, 1986
Euchrosia	Koopowitz, 1986
Rhodophilia	Koopowitz, 1986
Stenomesson	Meerow, 1989
Urceolina	Koopowitz, 1986

Table A.6.11: Occurrence of self-incompatibility in the Amaryllidaceae. Table lists genera in which self-incompatible species are found.

A note on the nomenclature of *Amaryllis belladonna*

The nomenclature of the cape belladonna lily, currently recognised as *Amaryllis belladonna*, has been, and continues to be, the subject of much conjecture. The following synonyms have been used:

Donna bella (Ferrari, 1633)
Amaryllis rosea (Lamarch, 1783)
Coburgia sp. (Herbert, 1819)
*Amaryllis belladonna** (Linn.) (Herbert, 1821)
Callicore rosea (Link, 1829)
*Belladonna purpurescens*** (Sweet, 1830)
Brunsvigia rosea

*The *A. belladonna* described by Linnaeus was what is currently known as *Hippeastrum equestre*, and of South American origin.
***Belladonna* was not conserved due to the use of *Belladonna* as a genus in the Solanaceae.

References: Uphof (1938); Traub (1983).